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Symposium on Cellular Resistance to Anticancer Drugs

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Introduction

Bruce A. Chabner,* Neil J. Clendeninn, and Gregory A. Curt[1]

The problem of drug resistance is encountered in two distinct clinical settings and, in each, it has devastating consequences for the patient. The first of these settings is the failure of initial chemotherapy, a frequent occurrence in patients with the more common solid neoplasms, but an unusual outcome in patients with hematologic malignancy. The second circumstance is the development of resistance to chemotherapy in patients who have initially responded but have then relapsed. In both instances, it is a common observation that tumors which are resistant to a plant alkaloid or antibiotic are often cross-resistant to other natural products, even though these other drugs have not been used in the patient's previous drug treatment. These findings now have their parallel in the several experimental tumors which exhibit pleiotropic drug resistance (PDR)[2] (1-3) to a wide range of structurally unrelated amphophilic compounds. Not only have these experimental tumors confirmed that agents quite dissimilar with respect to their structure and mechanism of action, including those listed in table 1, may share cross-resistance, but it has also been demonstrated repeatedly that any one of these drugs is capable of inducing cross-resistance to the other, dissimilar agents (4-7).

In more detailed cellular pharmacologic studies, the molecular basis for PDR has been examined. Cells exhibiting PDR accumulate lower intracellular concentrations of drug, although the reasons are unclear (8-10). In none of these experiments have actual drug uptake kinetics been examined, nor have the physical properties or intracellular location of binding to targets such as tubulin or DNA been analyzed. In fact, the process by which these agents enter and exit cells is still in some doubt. Some workers have suggested that the primary defect in PDR is an accelerated efflux of these drugs, and have shown that measures which interfere with the generation of ATP, such as glucose deprivation or azide, will increase intracellular drug levels (11,12). Taken together, the evidence for decreased drug accumulation and the pleiotropic nature of the resistance pattern suggest, but certainly do not prove, that the underlying defect(s) which accounts for PDR resides in the cell membrane.

It is thus logical that attention has turned to the cell membrane constituents of resistant cells. Kessel and Bosmann (13,14) were the first to demonstrate that dactinomycin-resistant cells developed altered membranes and altered membrane synthetic apparatus; they suggested that these changes might underlie the PDR commonly observed in dactinomycin-resistant cells. More recently, three groups have observed increased levels of a high molecular weight glycoprotein (150,000-180,000 daltons on SDS gels) in cell lines with PDR (4,9,15), although it remains to be established whether a single glycoprotein, such as the P170 protein described by Ling and his colleagues, is characteristic of the PDR phenotype or indeed plays an obligatory role in the process of resistance. In fact, Beck and Cirtain (16) demonstrated that inhibition of synthesis of membrane glycoproteins by tunicamycin or pronase digestion does not alter the resistant phenotype. Debenham et al (17) are thus far the only investigators to demonstrate that transfer of PDR to sensitive cell lines (by transfection with calcium phosphate precipitates of PDR cell DNA) is accompanied by transfer of the P170 protein. Other groups have found that PDR cells may have changes in membrane lipid composition. There appear to be alterations in intracellular proteins as well, as for example, the increased concentration of the V19 protein observed in vincristine-resistant Chinese hamster lung cells (*vide infra*). Thus far, there is no published information on the membrane or intracellular proteins of human tumor specimens, or human tumor cell lines which exhibit the PDR phenotype. If such a marker glycoprotein were, in fact, present on human malignancies which exhibit PDR, this protein would have importance as a target for monoclonal antibodies and as a predictive marker for drug resistance.

A second important characteristic of PDR cells is the reversal of resistance by calcium channel blockers. Verapamil has been shown to reverse resistance to vincristine in P388 cells (18,19), and to doxorubicin in Ehrlich ascites tumor cells (20). It is not clear that verapamil reversal is a common property of PDR cells; it may be characteristic of only a specific type of PDR. The bio-

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[2]As used in this manuscript, PDR refers to a pattern of resistance to agents of both the antibiotic and plant alkaloid classes, but may include drugs of alkylating type.

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TABLE 1.—Relative resistance of Chinese hamster cell lines to chemical agents

Agent	Relative resistance*	
	A†	B‡
Vincristine	650	—
Vinblastine	512	30
Mithramycin	441	—
Colchicine	350	180
Etoposide	139	—
Daunorubicin	131	76
Dactinomycin	125	—
Puromycin	105	100
Homidium bromide	67	—
Emetine	—	29
Demecolcine	28	—
Doxorubicin	—	25
Hydroxyurea	5.9	—
Melphalan	—	10
Mechlorethamine	—	3
6-Thioguanine	2.1	—
Chlorambucil	—	2.0
Cytarabine	1.9	1.0
Methotrexate	1.8	—
Carmustine	1.3	—
Bleomycin	—	1.0
5-FU	—	1.0
Cyclophosphamide	—	0.3
Deoxycorticosterone	—	0.1
1-Dehydrotestosterone	—	0.1

*Ratio of the concentration of drug required to inhibit growth in the mutant line, compared to the concentration necessary for this inhibition in the sensitive line.

†According to Biedler and Peterson (ref 4), for Chinese hamster cells resistant to vincristine.

‡According to Ling (refs 5-7), for Chinese hamster cells resistant to colchicine.

chemical basis of this reversal is poorly understood, in that calcium channels are known to exist only in excitatory tissues such as neurons, myocardium, or tissues derived from the neural crest. It is possible that this reversal is a consequence of the extreme hydrophobicity of verapamil, or its secondary effects on other ion channels. Whether verapamil or other calcium channel blockers could be used to overcome PDR in man is an exciting, but untested, possibility.

The papers which follow in this issue of *Cancer Treatment Reports* were delivered at the symposium on "Cellular Resistance to Anti-Cancer Drugs" organized by the Developmental Therapeutics Program of the Division of Cancer Treatment, National Cancer Institute. This conference was convened to consider the biologic and pharmacologic basis of PDR and the implications of this rapidly developing field for our drug discovery program. In particular, we posed the following questions to our discussants:

(*a*) Does a common biochemical change underlie PDR, or is the phenotype likely to be the result of a number of different mutations? Is there convincing evidence that alterations in drug uptake or efflux are responsible for PDR?

(*b*) Does PDR exist as a clinical finding and, if so, does it share features with PDR in experimental tumors?

(*c*) Should an experimental tumor model for PDR be incorporated into drug screening programs and, if so, at what stage? Will information concerning cross-resistance among natural products be helpful in the development of strategies to test new drugs?

(*d*) What treatment strategies currently on hand might be useful in preventing the appearance of PDR or in circumventing resistance once it occurs?

These and other questions will be addressed in the following papers, which represent the first collective effort to define the PDR syndrome, to describe its biochemical and genetic features, and to suggest strategies for its conquest in man.

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Drug Resistance in Chinese Hamster Lung and Mouse Tumor Cells[1]

June L. Biedler,[2,*] Tien-ding Chang,[3] Marian B. Meyers,[2] Robert H. F. Peterson,[3] and Barbara A. Spengler[3]

Studies of Chinese hamster and mouse cells with high levels of acquired resistance to dactinomycin, daunorubicin, or vincristine have shown that these different permeability mutants all display similar phenotypic alterations: dramatic changes toward normal cell morphology and growth behavior and substantially reduced oncogenic potential. All three resistant cell types show increased expression of a high molecular weight plasma membrane glycoprotein species, gp150. Uniquely, vincristine-resistant cells contain gene amplification-associated chromosome abnormalities (homogeneously staining regions or double minute chromosomes), and they oversynthesize a low molecular weight cytosolic protein (V19). Cells grown in the absence of drug are phenotypically unstable. Revertant cells decline in resistance, length or number of homogeneously staining regions or double minute chromosomes, and expression of gp150 and V19. These proteins are thus candidate products of amplified genes which may or may not be manifested cytogenetically. The phenomena of drug resistance and reverse transformation are currently being addressed in protein phosphorylation studies. [Cancer Treat Rep 67:859–867, 1983]

Studies in our laboratory over the past two decades have dealt with genetic mechanisms whereby somatic mammalian cells may develop resistance to cancer chemotherapeutic agents (in particular, methotrexate, dactinomycin, daunorubicin, and vincristine). The early observation that gave impetus to these studies was that, in antifolate-resistant mouse leukemia (1,2) and Chinese hamster lung (CHL) (3,4) cells characterized by increased levels of the target enzyme dihydrofolate reductase, there appeared to be specific metaphase chromosome alterations that were correlated with degree of resistance (5,6). Just as the karyotypic and biochemical findings had led us to suggest (5), the genetic mechanism for development of resistance to methotrexate associated with dihydrofolate reductase overproduction was demonstrated to be selective amplification of the gene encoding the target enzyme (7).

Amplified dihydrofolate reductase genes may be contained in a nonbanding, homogeneously staining region (HSR) of a chromosome, as we had hypothesized (5), and others have shown (8) by in situ hybridization methodology, or may reside in an abnormally banding region (ABR), revealed by trypsin-Giemsa staining techniques, with a distinctively deranged pattern of bands (6,9). The frequent occurrence of HSR or ABR in cells resistant to antifolate undoubtedly explains the specific chromosomal alterations in methotrexate-resistant mouse leukemia L1210 cells studied before the advent of chromosome banding technology (1,2). Amplified dihydrofolate reductase genes may also be carried on small, paired extrachromosomal bodies known as double minute chromosomes (DM), as found in many different methotrexate-resistant sublines (10). Thus, HSR, ABR, and DM are reliable indicators of dihydrofolate reductase gene amplification in a variety of antifolate-resistant Chinese hamster and mouse tumor cells.

In a similar line of investigation we have developed and studied CHL cell sublines with acquired resistance to dactinomycin (11), daunorubicin (12), and vincristine (13). Again, our approach was to obtain cell lines with high resistance levels to detect alterations in cell phenotype that could be directly related to development of resistance to one or another of these agents. However, as demonstrated earlier (11,12,14) and recently discussed

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(15), cells resistant to the three different agents are mutually cross-resistant and share an impaired capacity to take up (or retain) drug. These permeability mutants exhibit diverse phenotypic alterations that in most instances appear to relate to the plasma membrane, raising the question of whether a single genetic change can explain the multiplicity of phenotypic changes in cells selected with the three agents unrelated in mechanism of action and/or chemical structure. Our cytogenetic studies had been inconclusive (eg, ref 11) until we examined the most recently isolated CHL subline, DC-3F/VCRd, selected with a high concentration of vincristine. The notable finding was that these cells, unlike the CHL cells selected with dactinomycin or daunorubicin, were characterized by the presence of an HSR (15). As described in preliminary reports (16,17), subsequent examination of several different vincristine-resistant Chinese hamster and mouse tumor lines, and human cells as well, indicated that the cells resistant to this drug contained either HSR or DM.

The purpose of this report is to assess and compare phenotypic characteristics of cells resistant to dactinomycin, daunorubicin, and vincristine and to discuss whether the cytogenetic indication of gene amplification in vincristine-resistant cells is suggestive of an additional genetic alteration associated with this drug alone or whether development of resistance to dactinomycin and daunorubicin may likewise involve gene amplification without detectable cytologic consequence.

Characteristics of Drug-Resistant Cells

Chinese hamster DC-3F cells selected with high concentrations of either dactinomycin, daunorubicin, or vincristine exhibit approximately 1000-fold to 3000-fold increases in resistance to their selective agent and lower-level cross-resistance to the other two drugs (table 1). Similarly, the vincristine-resistant subline derived from mouse tumor MAZ cells is highly resistant to its selective agent and is cross-resistant to dactinomycin (table 1).

As described in detail in previous publications and shown in table 2, cells of all four sublines display a constellation of phenotypic alterations that distinguishes them markedly from the drug-sensitive, tumorigenic cells from which the resistant lines were derived. A general feature of our series of antibiotic- and alkaloid-resistant cells is that of "reverse transformation." During the course of drug exposure and development of resistant sublines of CHL cells, we noted striking alterations in morphology and in vitro growth behavior of the resistant cells. The parental, drug-sensitive DC-3F cells, established originally as the cell line Dede from normal CHL tissue and subsequently cloned, exhibit morphologic and growth properties associated with malignant cells in culture, are highly tumorigenic when tested in immunosuppressed host animals, and are thereby classified as spontaneous transformants. The drug-sensitive MAZ line was cloned from cells established in culture from a hydrocarbon-induced tumor of a C57BL/6 mouse; MAZ cells also behave as transformed cells in culture and are tumorigenic in various animal test systems. Drug-resistant murine cells, including MAZ/VCR (table 1), selected with dactinomycin, vincristine, or ethidium bromide, likewise gradually acquired morphologic and growth characteristics associated with normal cells in culture. These various observations led us to test the tumorigenic capacity of both drug-resistant and control cells; indeed, all of the drug-resistant cells tested, representing nine independently derived CHL or mouse sublines with acquired resistance to one or another of the four agents, showed reduced or abrogated oncogenic potential.

The phenomenon of reverse transformation, the patterns of cross-resistance, and the demonstration of reduced drug uptake (table 2) all suggested that the altered phenotype of the drug-resistant cells was mediated at least in part by the plasma membrane.

TABLE 1.—Characteristics of drug-resistant cell lines

Cell line	Drug (μg/ml)*	Relative resistance† to—				
		Dactinomycin	Daunorubicin	Vincristine	HSR/DM	V19‡
DC-3F		1	1	1	-	-
DC-3F/AD X	10	2450	54	1400	-	-
DC-3F/DM XX	10	263	883	285	-	-
DC-3F/VCRd-5L	50	267	178	2750	+	+
MAZ		1		1	±§	-
MAZ/VCR¶	20	571		3939	+	+

*Final selective concentration of dactinomycin (AD), daunorubicin (DM), or vincristine (VCR).

†Published (refs 11, 12, 15, and 18) and unpublished values.

‡Peptide (M_r 19,000; pI 5.7) visualized as a spot, after 2-dimensional gel electrophoresis of soluble proteins (ref 16).

§Parental, drug-sensitive MAZ cells typically contain variable numbers of DM in about 65% of cells. The mean No./cell and the frequency of DM-positive cells are markedly less than for the vincristine-resistant subline, MAZ/VCR.

¶Resistance levels were determined after 2-3 days of growth in the absence of drug in contrast to previously reported values (refs 15 and 17).

TABLE 2.—Phenotypic characteristics of dactinomycin-, daunorubicin-, and vincristine-resistant sublines of spontaneously transformed CHL and mouse tumor cells

Characteristic	Ref No.
Cross-resistance to a wide variety of agents, with a marked correlation between degree of resistance to, and molecular weight of, a chemical agent	11, 12, 15
Decreased permeability to drug	11, 12, 14
Cell morphology and growth behavior associated with that of normal (nontransformed) cells in culture	13, 14
Reduction or abrogation of tumorigenicity	13, 15

HSR and DM in Vincristine-Resistant and Revertant Cells

Early cytogenetic studies of dactinomycin-resistant CHL cells showed no evidence of specific chromosomal alteration associated with expression of the resistant phenotype (11). Nevertheless, when chromosome-banding methods became available, and in view of the specific chromosomal aberrations of antifolate-resistant cells (3–6), we examined the drug-resistant sublines listed in table 1. Trypsin-Giemsa banding analysis revealed that the vincristine-resistant CHL cells, but not those selected with either dactinomycin or daunorubicin, contain an HSR (15). The region is similar in appearance to the HSR of antifolate-resistant CHL sublines but has a different chromosomal location (15).

Vincristine-resistant MAZ/VCR cells (table 1) were found to contain extraordinarily large numbers of DM, with all cells examined having at least 50, and a quarter of the cells with > 200 DM/cell (17).

Earlier studies of antifolate-resistant CHL cells grown in the absence of drug had established a correlation between degree of resistance, level of dihydrofolate reductase activity, and mean length of the HSR in cells undergoing reversion to drug sensitivity (6). Furthermore, the revertant cells were demonstrated to have a reduced number of amplified dihydrofolate reductase genes (19). A similar study was therefore carried out with HSR-containing vincristine-resistant cells to determine whether they would undergo phenotypic reversion in the absence of drug and, if so, whether there was a relationship between level of resistance and HSR length. DC-3F/VCRd-5 cells, cloned from the DC-3F/VCRd subline selected at 10 μg/ml and exhibiting a 500-fold increase in resistance, gradually lost resistance when grown in drug-free medium (15,17). As shown in figure 1, there was a concomitant decline in the mean length of the HSR. Although individual cells differed in HSR length, the length decline was gradual rather than abrupt, and HSR-negative cells were not observed until near the end of the year-long experimental period.

DM-containing MAZ/VCR cells also lost resistance

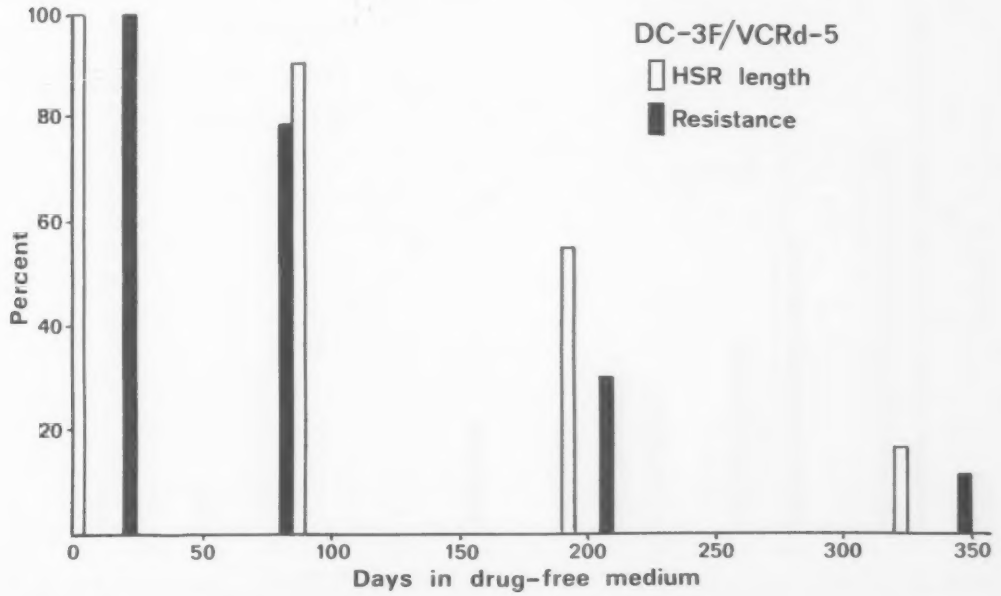

FIGURE 1.—Decrease in resistance and mean HSR length in DC-3F/VCRd-5 cells grown in drug-free medium. Results are expressed as the % of degree of resistance or of HSR length; 100% = 500-fold increase in resistance and an HSR length 0.68 as long as a normal Chinese hamster chromosome 2p arm.

when maintained in the absence of vincristine (17). Periodic determination of resistance level and mean number of DM per cell (fig 2) revealed a rapid decline in both parameters.

Thus, the vincristine-resistant CHL and mouse tumor cells, like their methotrexate-resistant counterparts (6,17), were phenotypically unstable when grown in drug-free medium. The concomitant, parallel decline in HSR length or DM number, moreover, suggests a relationship between expression of vincristine resistance and the presence of these gene amplification-associated chromosome abnormalities.

We also tested stability of the resistant phenotype in cell lines selected with dactinomycin or daunorubicin. Like the vincristine-resistant CHL cells, cells of the dactinomycin-resistant DC-3F/AD X cell line and of DC-3F/AD IV, a 376-fold resistant precursor line (11), gradually declined in resistance in the absence of drug (17), as did daunorubicin-resistant DC-3F/DM I cells (a precursor of the DC-3F/DM XX subline) with a 231-fold increase in resistance (J. L. Biedler and B. A. Spengler, unpublished observations). Thus, the phenotypic instability exhibited by HSR- and DM-containing sublines selected with antifolates (6,17) or vincristine (17) is also characteristic of the several antibiotic-resistant cell lines with no cytologic evidence of gene amplification.

Plasma Membrane Studies: Glycoproteins and Gangliosides

Since many of the phenotype alterations (table 2) associated with the development of resistance to dactinomycin, daunorubicin, and vincristine implicated the plasma membrane, we have attempted to identify possible differences between membrane components of drug-sensitive and drug-resistant cells (15,17,18,20,21).

To investigate plasma membrane composition, membranes were isolated from CHL cells metabolically labeled with ^{3}H-glucosamine, solubilized, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on cylindrical gels. The predominant glycoprotein synthesized by drug-sensitive DC-3F cells has an apparent molecular weight of 100,000, whereas cells selected with any of the three different agents express a high molecular weight M_r 150,000 glycoprotein designated gp150. Two-dimensional gel analysis confirmed this observation and showed that both the M_r 100,000 glycoprotein of parental DC-3F cells (and of drug-sensitive revertants) and gp150 of the various resistant cells comprise families of glycopeptides (18). Although there may be slight differences in gp150 among the different drug-resistant sublines, it is clearly the predominant glycoprotein species in cells resistant to vin-

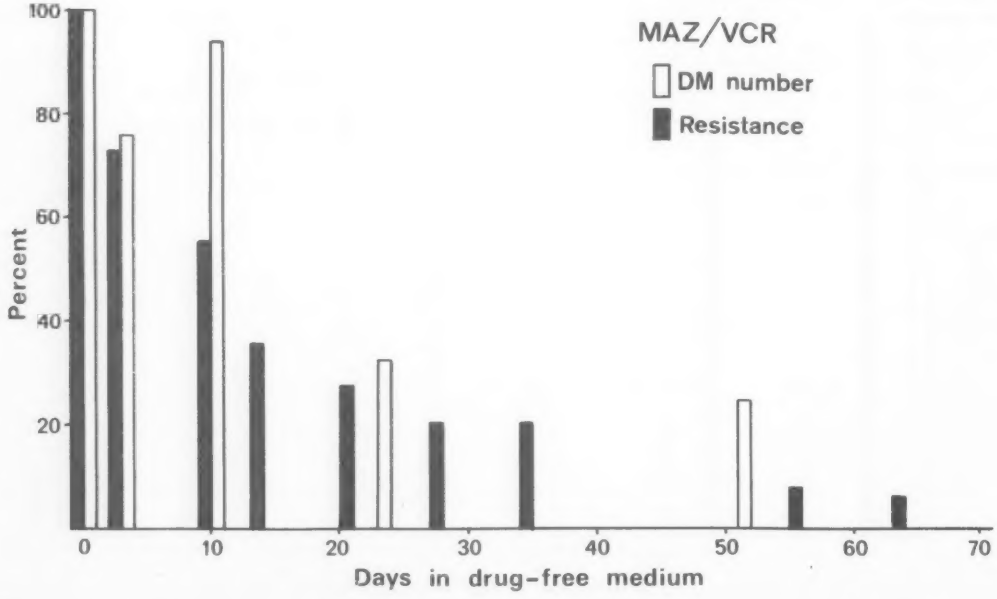

FIGURE 2.—Decrease in resistance and mean No. of DM in MAZ/VCR cells grown in drug-free medium. Results are expressed as % of degree of resistance or of DM No. characteristic of drug-treated MAZ/VCR cells.

cristine, dactinomycin, or daunorubicin. Quantitative analyses of the relative amounts of the major membrane glycopeptide species visualized in the cylindric SDS-PAGE studies revealed that the only consistent differences were in the relative proportions of gp150 and the M_r 100,000 species (15,18). Moreover, as shown in figure 3, there was a clear correlation between the increase in expression of gp150, the decrease in expression of the M_r 100,000 species, and the increase in resistance or cross-resistance to dactinomycin.

Loss of resistance during growth in drug-free medium was associated with changes in expression of the two glycoprotein species. Revertant DC-3F/AD X cells (fig 3), exhibiting a 45-fold reduction in resistance to dactinomycin, and revertant DC-3F/VCRd-5 cells, with a 15-fold decrease in resistance to vincristine, now contained only trace amounts of gp150 and had regained expression of the M_r 100,000 glycopeptide, with amounts similar to those found in drug-sensitive DC-3F cell plasma membranes (18).

Gangliosides represent another category of plasma membrane components which, in view of their involvement in a wide variety of membrane-related phenomena, were also subjected to analysis. In initial studies (21) we examined drug-sensitive DC-3F cells, the 2450-fold dactinomycin-resistant DC-3F/AD X subline (table 1), and the revertant subline grown in the absence of drug for 3 years and exhibiting a level of resistance only 50-fold higher than control. Control cells express a full complement of gangliosides from hematosides (G_{M3}) to disialogangliosides (G_{D1a}), with hematosides being the least abundant species. The dactinomycin-resistant cells, in contrast, appear to express only hematosides. Revertant cells regained full expression.

We next examined the ganglioside composition of daunorubicin-resistant DC-3F/DM XX cells (table 1) and the vincristine-selected subline DC-3F/VCRd with a 650-fold increase in resistance (15). The 883-fold daunorubicin-resistant cells, like the dactinomycin-resistant DC-3F/AD X subline, were found not to synthesize higher gangliosides except for trace amounts of G_{D1a}. The 650-fold vincristine-resistant cells are, on the other hand, similar to DC-3F control cells in expressing a full range of gangliosides. Since a dactinomycin-resistant

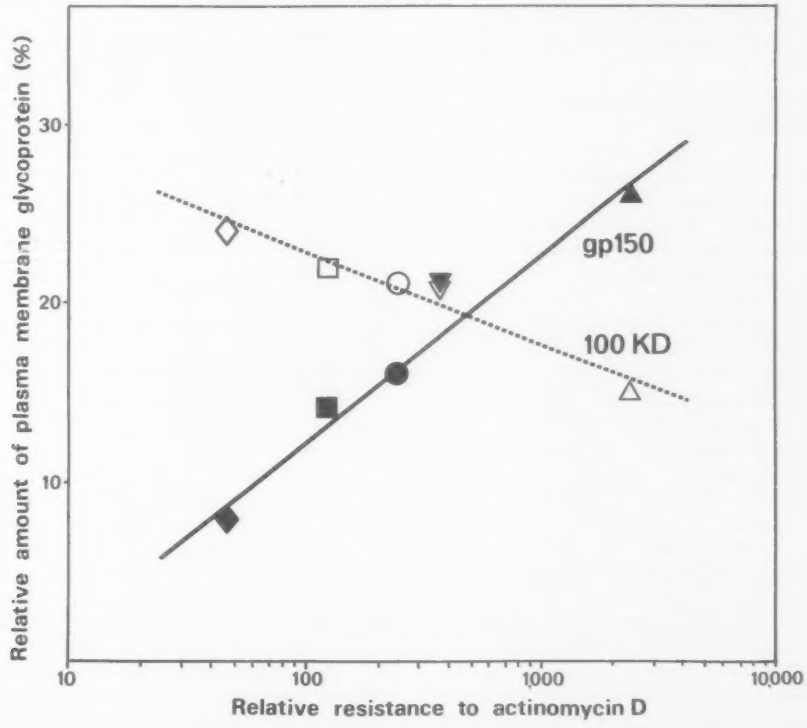

FIGURE 3.—Relative increase in gp150 and decrease in M_r 100,000 glycoprotein species correlated with increase in resistance or cross-resistance to dactinomycin. ◇◆ = revertant DC-3F/AD X-U; □■ = DC-3F/VCRd; ○● = DC-3F/DM XX; ▽▼ = DC-3F/AD IV; △▲ = DC-3F/AD X; open symbols = relative amount of 100 KD glycoprotein; solid symbols = relative amount of gp150. Percents were calculated from SDS-PAGE gels of purified, radioactively labeled membrane preparations, as described elsewhere (20).

CHL cell subline (DC-3F/AD IV) with a lower level of resistance (376-fold) than the initially examined DC-3F/AD X subline (table 1) had, as did revertant cells, a pattern of ganglioside expression like that of drug-sensitive control cells, we suggested that there may be a threshold effect: resistance of approximately 1000-fold or greater to any one of the three agents would result in a simplified ganglioside biosynthetic pattern (15). Recent analyses of the newly derived 2750-fold vincristine-resistant DC-3F/VCRd-5L subline (table 1) have shown, however, that this is not the case; these highly resistant cells synthesize a full complement of monogangliosides and disialogangliosides, with relatively more G_{M3} and G_{M2} (18).

Thus, in the CHL cell system, development of resistance to dactinomycin, daunorubicin, or vincristine leads to similar plasma membrane glycoprotein alterations but differing changes in ganglioside synthesis. It is not possible to interpret these findings at this time, nor is it possible to understand whether or how the membrane alterations relate to altered phenotypic expression of drug resistance and/or malignancy. Nevertheless, the differential ganglioside profile of vincristine-resistant cells, as compared to dactinomycin- and daunorubicin-resistant cells, raises the possibility of genetic differences between cells resistant to the vinca alkaloid and the antibiotics.

Low Molecular Weight Cytosolic Protein of Vincristine-Resistant Cells

Because of our findings that a number of different vincristine-resistant cell lines, including DC-3F/VCRd-5L and MAZ/VCR (table 1), selected from Chinese hamster, mouse, or human cell populations, show gene amplification-associated cytogenetic abnormalities, we have undertaken a search for a putative product of amplified genes via several different approaches. Initial screening of cell proteins by SDS-PAGE was unrewarding, for, unlike HSR-containing antifolate-resistant DC-3F cells with amplified dihydrofolate reductase-encoding genes, where the overproduced enzyme is readily visualized by this technique (22), there were no immediately discernible differences between control and vincristine-resistant Chinese hamster cells. However, two-dimensional gel electrophoretic analyses of cell proteins revealed the presence of a low molecular weight M_r 19,000 acidic protein (pI 5.7), designated V19, that is consistently present in the supernatant fractions and not detectable in the pelleted membranous fractions of vincristine-resistant DC-3F/VCRd-5 and MAZ/VCR cells (16). Only trace amounts of V19 are synthesized by parental DC-3F or MAZ cells. Vincristine-resistant human neuroblastoma cells oversynthesize apparent counterpart low molecular weight peptides (16). Decrease of resistance in revertant CHL and mouse tumor cells grown in the absence of vincristine (figs 1 and 2) was accompanied by a decrease in intracellular amount of V19 (16,17). These observations and the correlation between the increased synthesis of V19 in HSR- and DM-containing vincristine-resistant cells and not in the dactinomycin- and daunorubicin-resistant cells tested so far (table 1) implicate this peptide as a candidate product of amplified genes.

Phosphorylation Studies

We are currently assessing our findings that vincristine-resistant as well as dactinomycin- and daunorubicin-resistant cells undergo phenotypic reversion toward normal in vitro and in vivo growth behavior, in light of similar demonstrations of short-term reverse transformation associated with elevated intracellular levels of cAMP. For example, Lockwood et al (23) have suggested that the spectrum of phenotypic changes mediated by cAMP in transformed cells in culture is due to cAMP-dependent protein kinase activity which acts to phosphorylate and thereby alter a variety of cellular proteins. We have initiated investigations of protein phosphorylation in vincristine-resistant and control cells.

Incubation of cytosols from drug-resistant and control cells with [γ-^{32}P]ATP in the presence or absence of cAMP followed by SDS-PAGE analysis of labeled proteins showed that the overall level of protein phosphorylation is higher in the resistant cells (M. B. Meyers et al, unpublished data). Furthermore, there are increases and decreases in the extent of phosphorylation of individual proteins when vincristine-resistant and control cells are compared. As shown in figure 4, one of the notable differences in phosphoprotein pattern is the increased phosphorylation of an M_r 18,700 protein in cytosols from MAZ/VCR cells. Another difference is the phosphorylation of V19, which is enhanced in the presence of cAMP under the conditions of these experiments. Phosphorylation of V19 has not been observed in cytosols of control MAZ cells. Similar results were obtained in studies of DC-3F and DC-3F/VCRd-5L cells, ie, increased phosphorylation of an 18.7 KD peptide and increased phosphorylation of V19 in the presence of cAMP, in vincristine-resistant cell cytosols.

As described in a preliminary report (24), low molecular weight protein fractions from vincristine-sensitive and -resistant cell cytosols were obtained by BioGel P-60 chromatography to investigate specific phosphorylation activity of V19 and the M_r 18,700 protein. V19-containing fractions were enriched in that protein by tenfold to 20-fold. In vitro phosphorylation studies with the low molecular weight material from either DC-3F or MAZ cells and their respective vincristine-resistant sublines demonstrated that the major phosphorylated protein in that molecular weight range was the 18.7 KD species. V19 was not phosphorylated under these conditions. Phosphorylation of the 18.7 KD protein in resistant cells was increased at least tenfold compared to control. The cytosol fractions from resistant cells also

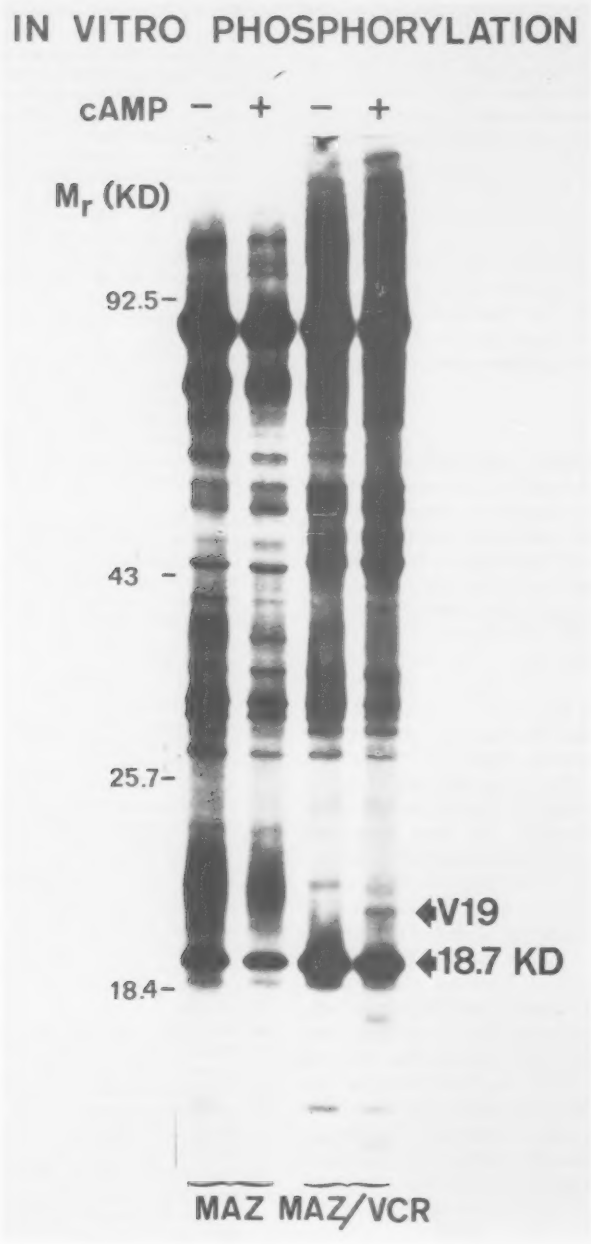

FIGURE 4.—Cytosol proteins from drug-sensitive MAZ and vincristine-resistant MAZ/VCR cells, phosphorylated in vitro with [γ-^{32}P]ATP in presence or absence of cyclic AMP and separated by SDS-PAGE. Arrows indicate the resistance-related V19 peptide and a 2nd peptide, with an apparent molecular weight of 18,700, which exhibits increased phosphorylation in MAZ/VCR cytosols.

had histone and casein kinase activity, whereas that of drug-sensitive Chinese hamster and mouse cells had little or no activity. The putative kinase activity in low molecular weight protein material from vincristine-resistant cells is also of interest in view of the recent finding that gp150, the predominant plasma membrane glycoprotein of DC-3F/VCRd-5L cells, is phosphorylated in vivo (R. H. F. Peterson et al, unpublished data). In contrast, the M_r 100,000 species characteristic of drug-sensitive CHL cells is apparently not phosphorylated. These various experiments demonstate that there are important phosphorylation differences between vincristine-resistant and control cells. The alterations observed in the resistant cells may be major factors in the drug-resistant and/or the reverse transformation phenotype.

DISCUSSION

The spectrum of phenotypic changes outlined in table 2 as well as the observed similarities in plasma membrane glycoprotein phenotypes suggests a common genetic mechanism(s) whereby CHL and mouse tumor cells develop resistance to the three different agents, dactinomycin, daunorubicin, and vincristine. On the other hand, vincristine-resistant cells appear to have several unique attributes. The resistant CHL cells do not show a major change in ganglioside expression. Vincristine-resistant hamster and mouse cells oversynthesize a cytosolic protein, V19, and have gene amplification-associated cytogenetic abnormalities. These findings raise the possibility that the vincristine-resistant cells have a different, additional genetic lesion(s). The notion that loss of resistance in the absence of selection pressure is indicative of a gene amplification mechanism has been considered (6,10,17). All of the variously resistant sublines that we have studied show phenotypic instability in the absence of drug so that, with time, resistance declines as does the synthesis of plasma membrane gp150 and, in vincristine-resistant cells specifically, the synthesis of V19 and the size or number of HSR and DM. Therefore, either an unstable phenotype is not a reliable indicator of gene amplification or, in dactinomycin- and daunorubicin-resistant cells, gene amplification has occurred without cytologic manifestation. Thus, at this point in our studies, both gp150 and V19 may be considered products of amplified genes. In any case, we anticipate that the ongoing studies of protein phosphorylation will aid in elucidating the possible relationship between these two phosphoproteins and their role in drug resistance and/or reverse transformation.

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Multidrug-Resistance Phenotype in Chinese Hamster Ovary Cells[1]

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Multidrug resistance is a complex pleiotropic phenotype of cross-resistance and collateral sensitivity to unrelated compounds observed in many mammalian cell mutants selected for resistance to single agents. In Chinese hamster ovary cells, colchicine-resistant mutants expressing this phenotype have been characterized extensively. Such mutants arise apparently from a single genetic event, and the basis of this phenotype appears to be localized at the membrane level, resulting in altered drug permeability. Expression of a 170,000-dalton surface glycoprotein (P-glycoprotein) has been identified to correlate with the multidrug-resistance phenotype. Selection of a second mutation in colchicine-resistant mutants, for resistance to phytohemagglutinin, results in an alteration of the carbohydrate moiety in P-glycoprotein and other surface components. This mutation does not noticeably affect the multidrug-resistance phenotype. The altered permeability of mutant cells to drugs, however, can be modulated by nonionic detergents or metabolic inhibitors. These findings are consistent with a molecular mechanism of multidrug resistance whereby the pleiotropic response of the cell is mediated by an overexpression of a cell-surface protein, the P-glycoprotein.
[Cancer Treat Rep 67:869–874, 1983]

In the progression of neoplastic diseases to advanced cancers, resistance to combination chemotherapy is often encountered. One possible basis for this nonresponse is that mutations resulting in concomitant resistance to a combination of drugs occur in the malignant stem cells, and that on application of therapy, such variants survive to become a dominant component of the disease. Studies in model systems, for example in Chinese hamster ovary (CHO) tissue culture cells, indicate that selection for variants resistant to single agents often yields cells expressing resistance to unrelated compounds, ie, a multidrug-resistance phenotype. These variants occur spontaneously at frequencies of 10^{-7}–10^{-5}, depending on the drug and selection conditions used. Multidrug resistance is observed in CHO cells selected for resistance to the following drugs: dactinomycin, doxorubicin, colchicine, cytochalasin B, demecolcine, daunorubicin, emetine, epipodophyllotoxin, podophyllotoxin, taxol, and vincristine. Since similar findings have been noted in other systems (1,2), it seems likely that mutations would also occur in human neoplasms (albeit at a frequency not yet defined), leading to the emergence of a therapy-resistant disease.

Multidrug-Resistance Phenotypes

One question of interest is: how different are the multidrug-resistance phenotypes in resistant lines selected under different conditions? This question was approached by comparing colchicine-resistant (CH^R) and daunorubicin-resistant (DNR^R) clones derived from the same parental CHO cell line. Selections for such clones were undertaken as shown in figure 1, and the response of the highly resistant clones to a variety of compounds is shown in table 1.

In both cases, extensive cross-resistance to a variety of unrelated drugs is expressed, although the degree of resistance to particular drugs is different. For example, among the drugs listed in table 1, the CH^R line was most resistant to colchicine and its response to daunorubicin ranked third, while the DNR^R line was most resistant to

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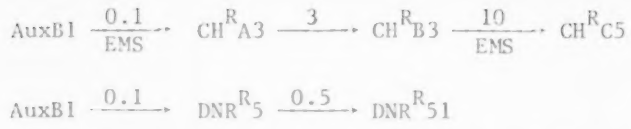

FIGURE 1.—Stepwise clonal selection for colchicine or daunorubicin resistance in CHO cells. Selected drug concentrations in μg/ml are as indicated. Prior treatment with mutagen EMS (ethyl methanesulfate) was undertaken in some cases.

daunorubicin and its response to colchicine ranked fourth. Thus, it appears that the specific multidrug-resistance phenotype can be very complex. In this context it is noteworthy that increased sensitivity (collateral sensitivity) is displayed by the CH^R line to some compounds such as acronycine and Triton X-100 (table 1). The response of such mutants to a series of related, nonionic Triton X detergents further highlights the complexities of this phenotype, in that it spans from cross-resistance to collateral sensitivity, depending on the number of ethylene oxide residues in the side chain of the detergent molecule. Thus, CH^RC5 is cross-resistant to Triton X-165 with a chain length of 16 residues but is collaterally sensitive to Triton X-102 with a chain length of 12-13 residues (3).

The above findings have two implications for cancer chemotherapy, at least at the conceptual level. First, by analogy with the observation that quite different multidrug-resistance phenotypes can be expressed in model systems, it seems likely that putative tumor cells bearing the multidrug-resistance phenotype may display a wide range of response, depending on the history of the disease and treatment protocol used. Thus, it may not be possible to model adequately a priori the cross-resistance pattern of a particular neoplasm. Given such a situation, optimal drug combinations to be used for treatment would have to be established individually from the drug-sensitivity profile of each patient's disease. Second, the observation of collateral sensitivity is intriguing. At this point the molecular basis for this phenotype is not known; nevertheless, this observation may be exploitable in some cases in formulating drug combinations for therapy.

TABLE 1.—Multidrug resistance in CH^RC5 and DNR^R51

Drug	Relative resistance*	
	CH^RC5	DNR^R51
Colchicine	180	25
Puromycin	~100	38
Daunorubicin	76	41
Taxol	~20	~5
Vinblastine	30	22
Emetine	29	11
Doxorubicin	25	~30
Melphalan	4-15	NA
Deoxycorticosterone	0.1	NA
1-Dehydrotestosterone	0.1	~1.0
Acronycine	< 0.06	0.6
Triton X-100	0.3	NA

*Relative resistance was determined by the concentration of drug required to inhibit growth or colony formation in the drug-resistant line compared with the parental line. NA = not analyzed.

Genetic Analysis

Genetic characterization of the multidrug-resistance phenotype was undertaken in some detail in the CH^R CHO cells (1-8). Findings from these studies fully support the conclusion that this complex phenotype results from a single genetic alteration or event. Some of the key observations are summarized as follows: (*a*) independent, single-step, clonal isolates obtained with or without prior mutagen treatment all yielded variants displaying multidrug resistance; (*b*) selection for increased resistance to single agents, such as colchicine or daunorubicin, results in increased multidrug resistance; (*c*) cell-cell hybrid studies indicate that both cross-resistance and collateral sensitivity are expressed in a dominant (incomplete) manner; (*d*) independent revertants selected for increased sensitivity to puromycin or decreased collateral sensitivity to 1-dehydrotestosterone have reduced multidrug resistance; and (*e*) DNA-mediated gene transfer of colchicine resistance into drug-sensitive mouse cells, using DNA from CH^R CHO cells, results in transformants that express both colchicine resistance and multidrug resistance. Unlinked genes, such as those coding for thymidine kinase, for ouabain resistance, and for methotrexate resistance are not co-expressed in such a system.

Thus, the complex phenotype displayed by multidrug resistance is likely the result of a single mutation with pleiotropic effects rather than multiple mutations. This latter conclusion and the fact that the phenotype is dominantly expressed imply that such a mutation can be expressed in a variety of cells including fully diploid normal cells or polyploid malignant cells.

Localization of the Multidrug-Resistance Mutation at the Plasma Membrane: Expression of P-Glycoprotein

Early studies of the CH^R lines indicated that the mutants accumulated labeled colchicine at a reduced rate compared with the parental cells and that this correlated with the degree of drug resistance (7). The interaction of colchicine with its cytoplasmic target protein, tubulin, was not affected (7,9). Moreover, uptake studies with

other drugs to which the CH^R lines displayed cross-resistance showed that those drugs were also accumulated at reduced rates (8). It was concluded, therefore, that the multidrug-resistance phenotype results from a pleiotropic mutation effected at the plasma membrane level.

Examination of the plasma membranes of the CH^R lines by gel electrophoresis indicated that an increased expression of a cell-surface glycoprotein of about 170,000 daltons, called the P-glycoprotein, correlates with the degree of drug resistance in independent colchicine mutants, hybrid cell lines, and revertants (1,2,10).[5] Development of antisera against this cell-surface antigen has provided a sensitive and specific means of detecting P-glycoprotein expression in DNA-mediated transformants (5). Such antisera were used to reveal increased expression of P-glycoprotein in CHO cells selected for resistance to other drugs, such as daunorubicin (fig 2) and vincristine (unpublished observation). Moreover, multidrug-resistant lines from mouse and human all display an increased expression of a 170,000-dalton surface component which was cross-reactive with the P-glycoprotein of the CH^R cells by immunoblotting analysis.[5] Both the concordance of molecular size and immunocross-reactivity of the P-glycoprotein displayed by multidrug-resistant lines from different species indicate that P-glycoprotein is a conserved cell-surface antigen. Taken together, all of the above findings provide a strong basis for the conclusion that the expression of P-glycoprotein is intimately associated with the multidrug-resistance phenotype and that it plays a key role in mediating this pleiotropic phenotype.

Whether or not increased expression of the surface P-glycoprotein alone is sufficient to account for all complexities of the multidrug-resistance phenotype is not known at present, since a variety of other membrane-associated changes have been found in different drug-resistant lines (11,12) and alterations in cytoplasmic proteins have also been implicated (13). Efforts to isolate the P-glycoprotein gene are currently underway, and the availability of a cloned P-glycoprotein gene coupled with gene transfer should provide an approach to answer this question more definitively. Such a reagent should also facilitate investigation of the molecular basis of increased P-glycoprotein expression. Our current speculation is that this is the result of gene amplification (1).[6]

Uptake of Colchicine

A detailed study of labeled colchicine accumulation by whole cells has indicated that colchicine enters cells via kinetics consistent with an unmediated diffusion process (14,15). Properties such as lack of competition with the close analog colcemid, inability to demonstrate either saturation kinetics or inhibition by sulfhydryl inhibitors or by ouabain, and stimulation by nonionic detergents are all consistent with this conclusion. Of interest is the observation that in the absence of a sugar energy source, eg, glucose, metabolic inhibitors such as cyanide or azide stimulated the accumulation rates of labeled colchicine so that the difference between drug-resistant and drug-sensitive cells which is observed in the untreated cells is not evident. In the presence of glucose under the same conditions, no stimulation is observed, and the rate of drug accumulation in the mutant cells is much reduced compared to wild-type. The basis of this stimulation is not understood, although it may be associated with the level of intracellular ATP (16).

In studies of anthracycline-resistant lines, Danϕ (17) has postulated an increased energy-dependent efflux mechanism as the basis of the observed reduced daunorubicin accumulation. Whether or not such a mechanism could account for the colchicine-resistance phenotype has not been studied in detail; however, two observations are relevant to this question. First, colchicine taken up into whole cells binds very tightly to microtubule protein, and free colchicine is not normally present (7). Thus, for an efflux mechanism to account for reduced labeled colchicine accumulation, it must operate at a site (at the cell surface?) prior to the drug's encounter with the binding protein. Second, the activation energy of colchicine accumulation in CHO cells under normal conditions, or with detergent-stimulated or metabolic inhibitor-stimulated cells, yielded values between 19 and 20 KCal/mol (14,15), indicating that the mechanism of drug accumulation under these different conditions is basically the same. These results we believe are consistent with a dynamic model where the plasma membrane provides an *active barrier* modulating the diffusion of a variety of molecules into and out of the cell. It seems possible that an overexpression of the cell-surface P-glycoprotein in mutant cells could perturb this modulation so that a complex pleiotropic phenotype of multidrug resistance results. Such a model could also easily accommodate the wide variations observed in the multidrug-resistance phenotype since such variations could presumably be generated by slight differences in the composition and/or organization of membrane components.

Mutation to Phytohemagglutinin Resistance (Pha^R)

Another genetic approach has been taken to probe the role of cell-surface components in modulating the multidrug-resistance phenotype. We selected for a second mutation, resistance to the lectin phytohemagglutinin (Pha), in the CH^RC5 mutant line, thereby generating a double mutant carrying both the CH^R and Pha^R markers. Previ-

[5]Kartner N, Riordan JR, and Ling V. Cell surface P-glycoprotein is associated with multidrug resistance in mammalian cell lines. In press. *Science*, 1983.

[6]Robertson SM, Ling V, and Stanners CP. Co-amplification of double minute chromosomes, multidrug-resistance, and cell surface P-glycoprotein in DNA-mediated transformants of mouse cells. Manuscript submitted to *Molecular Cell Biology*, 1983.

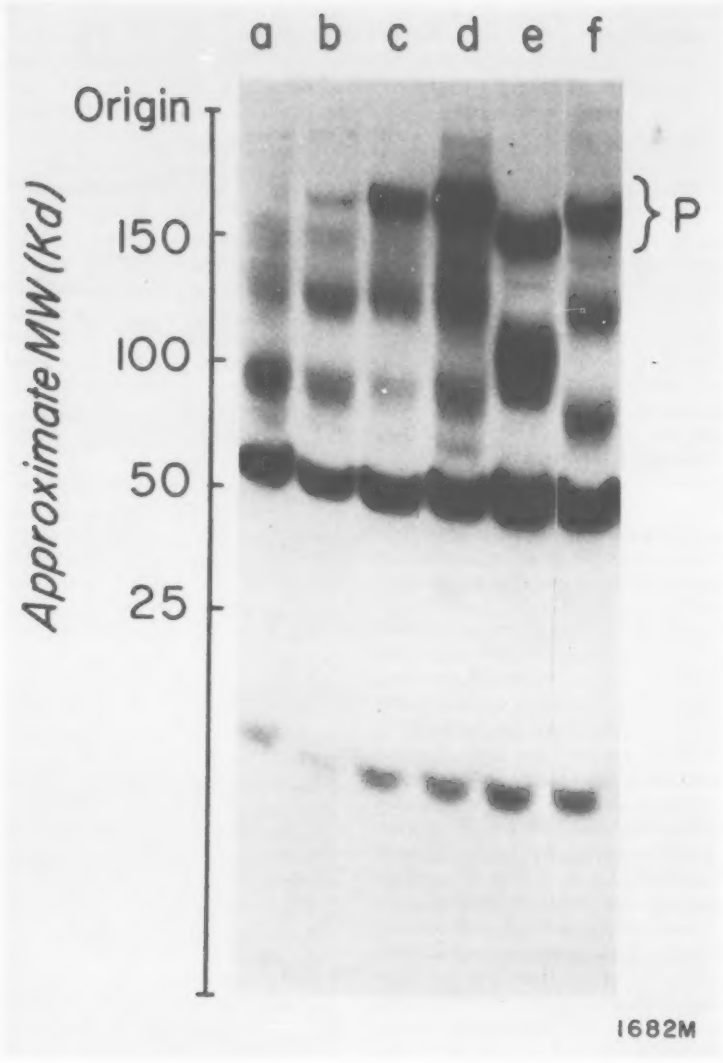

FIGURE 2.—Immunoblot analysis of membrane components. Plasma membranes were prepared from various lines as previously described (ref 10) and 50 μg of membrane protein/lane was fractionated by sodium dodecyl sulfate gel electrophoresis. Fractionated components were transferred to nitrocellulose paper and stained with ^{125}I-labeled protein A and rabbit antiserum raised against CH^{R}C5 membrane, in a Western blot procedure (refs 5,8). Lane (a) AuxB1, (b) CH^{R}A3, (c) CH^{R}B3, (d) CH^{R}C5, (e) CP-3.7, and (f) DNR^{R}51. *See* fig 1 for origin of the various lines.

ously, Baker and Ling (2) and Stanley and Siminovitch (18) have shown that selection for Pha^{R} in CHO cells frequently yielded a class of mutants (Class I) with greatly reduced surface carbohydrate as a result of a defect in *N*-acetylglucosaminyl transferase activity. A number of CH^{R} Pha^{R} double mutants were isolated and one of them (CP-3.7), with properties characteristic of a Class I Pha^{R} mutant, was studied in greater detail as described below.

Incorporation of labeled glucosamine by CP-3.7 cells was reduced to about 25% of that observed in CH^{R}C5 cells, indicative of a Class I mutation. Analysis of the P-glycoprotein in CP-3.7 cells showed that it migrates at a significantly lower molecular weight, about 140,000 (fig 2, lane e), indicating that the P-glycoprotein had a substantial loss of carbohydrate (compare lanes d and e in fig 2). Changes in the mobility of other cell-surface com-

ponents were also observed as a result of the Pha^R mutation. All of these data are indicative of a significant alteration in the carbohydrate content of CP-3.7 cells compared with CH^{R}C5 cells.

Analysis of the drug-resistance phenotype is shown in figures 3 and 4. Figure 3B shows that the CP-3.7 line is highly resistant to Pha and that the response of CH^{R}C5 cells is similar to the parental drug-sensitive line. The acquisition of the Pha^R mutation did not affect the colchicine-resistance phenotype (fig 3A), nor did it affect the degree of cross-resistance to daunorubicin and puromycin (fig 4, A and B). Collateral sensitivity to 1-dehydrotestosterone was also not affected (data not shown). Thus, it appears that the Pha^R marker, which resulted in a major alteration of the carbohydrate component of P-glycoprotein and other surface components, does not affect the multidrug-resistance phenotype. This is a remarkable observation, and we can only conclude that cell-surface carbohydrate and, in particular, the carbohydrate moiety of the P-glycoprotein, does not play a major role in modulating multidrug resistance. This conclusion is in general agreement with the observation of Beck and Cirtain (19) who showed that removal of cell-surface glycopeptides by pronase digestion and inhibition of glycosylation with tunicamycin did not significantly affect the expression of vinblastine resistance in a multidrug-resistant human line.

CONCLUSIONS

The study of multidrug resistance in the well-characterized CH^R CHO cells has provided us with both conceptual and practical tools to further tackle the more complex problem of therapy resistance in advanced cancers. Using the CHO cell system as a model, it is clear that multidrug resistance has a genetic basis, that it could arise in human neoplasms by mutations, and that therapy-resistant malignant cells could emerge and ultimately dominate the disease. The multidrug-resistance phenotype is extremely complex, with many variations, but our studies to date indicate that an increased expression of a conserved cell-surface antigen, the P-glycoprotein, is invariably associated with this phenotype. Further studies will be required to extend the generality of this observa-

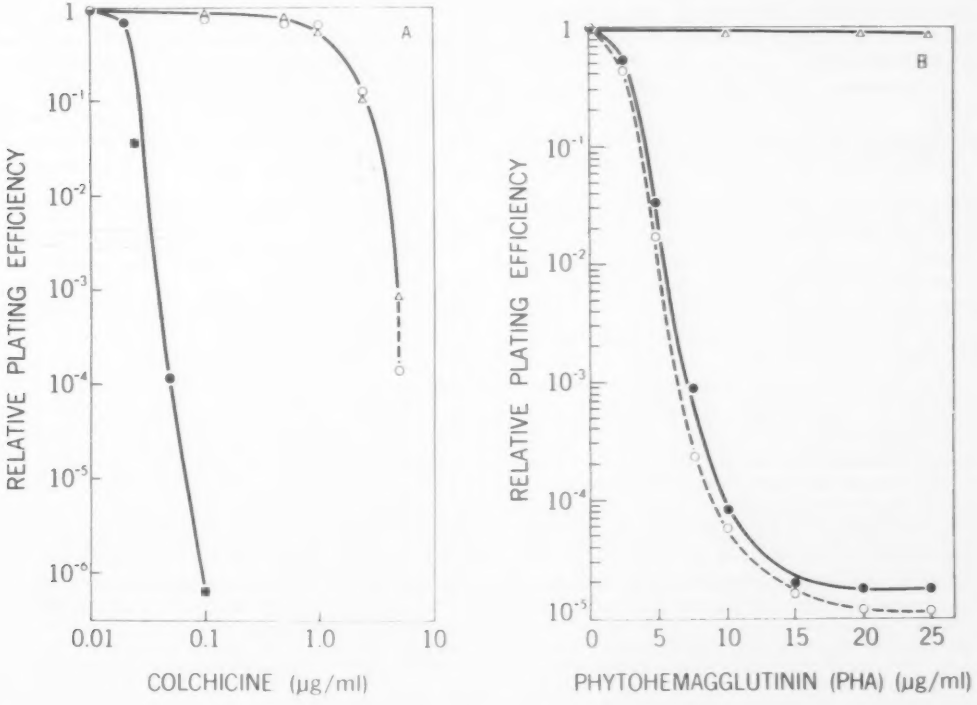

FIGURE 3.—Relative plating efficiencies of AuxB1, CH^{R}C5, and CP-3.7 cells. Cells from exponentially growing suspension cultures were plated at various cell numbers (10^2–10^5 cells/plate) in the presence of different drug concentrations. After incubation for 8–10 days at 37°C, colonies formed were stained with 1% methylene blue, counted, and the relative plating efficiency of each line was calculated. •——• = AuxB1; ○----○ = CH^{R}C5; and △- - -△ = CP-3.7.

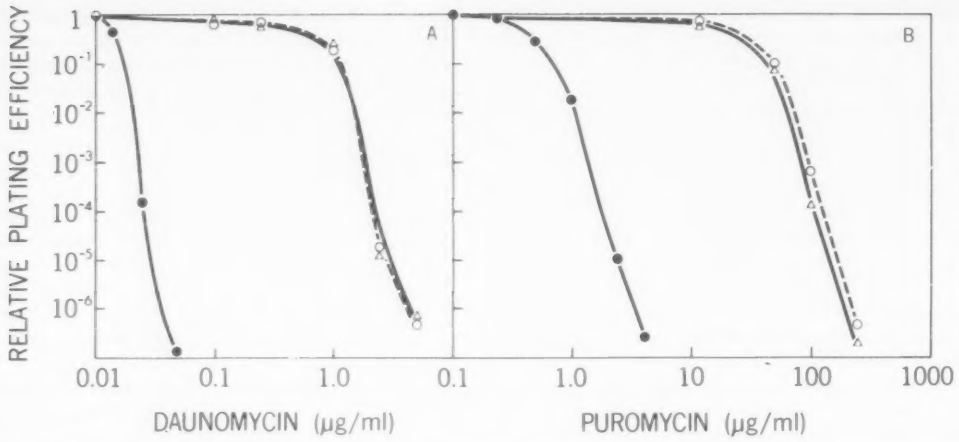

FIGURE 4.—Cross-resistance of CH^RC5 and CP-3.7 cells. Relative plating efficiencies in daunorubicin (daunomycin) and puromycin were measured as described in fig 3. •——• = AuxB1; ○- - -○ = CH^RC5; and △——△ = CP-3.7.

tion to therapy-resistant tumors. It seems possible, therefore, that the detection of P-glycoprotein expression may be exploited for diagnostic and therapy applications. Work is currently ongoing to generate immunologic reagents for this purpose.

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Vinca Alkaloid-Resistant Phenotype in Cultured Human Leukemic Lymphoblasts[1]

William T. Beck[2,3,*]

Resistance toward many "natural product" antitumor drugs, and its defining biochemical and pharmacologic lesions, has been demonstrated both in vivo (1-4) and in vitro (5-10) in several animal tumor models. This phenomenon, however, has not been studied extensively in human-derived tumor cells. In fact, distinct biochemical lesions associated with clinical resistance (to methotrexate) have only begun to appear (11). Over the past several years, work in my laboratory has been concerned with the phenotypic expressions of vinca alkaloid resistance in cultured human leukemic lymphoblasts (12-15) in order to understand the molecular basis of multiple-drug resistance. Summarized here are some of our observations describing the vinca alkaloid-resistant phenotype in these cells. The molecular changes that have been identified may ultimately serve as biochemical markers of drug-resistant cells in patients.

Alkaloid-Resistant Human Leukemic Cells and Multiple-Drug Resistance

Beginning with the T-cell lymphoblastic cell line, CCRF-CEM, established in culture from a patient with acute lymphoblastic leukemia (16), we selected cells for resistance to the vinca alkaloids vinblastine (VLB) (12) and, more recently, vincristine (VCR) (15). VLB-resistant cells were initially chosen because of the relatively greater commercial availability and chemical stability of [^{3}H]VLB, compared to [^{3}H]VCR; there appear to be no qualitative differences in the expressions of resistance and cross-resistance of our alkaloid-resistant sublines, CEM/VLB$_{100}$ and CEM/VCR$_{100}$. These alkaloid-resistant human lymphoblasts display the multiple-drug resistance phenotype, as has been demonstrated in alkaloid- or antibiotic-resistant mouse and hamster lines (5-10) (table 1). Their cross-resistance extends not only to the other vinca compounds, but also, although to a lesser extent, to other "natural products" with considerably different structures, viz, colchicine, anthracyclines, dactinomycin, and epipodophyllotoxins. Interestingly, these cells are not (or are only minimally) cross-resistant to podophyllotoxin, the parent compound of etoposide. We have made similar observations with our VCR-resistant cells (15). Thus, it appears that human leukemic lymphoblasts are capable of expressing the multiple-drug resistant or "pleiotropic" (9) phenotype. It should be emphasized, however, that this occurs at high degrees of alkaloid resistance, and may not be relevant to clinical drug resistance.

Cellular and Molecular Features of Vinca Alkaloid Resistance in Human Leukemic Lymphoblasts

We showed that our CEM/VLB$_{100}$ cells exhibited an altered culture morphology, growing in stationary suspension culture as large, raspberry-like aggregates, unlike the single-cell suspensions characteristic of the drug-sensitive parent CEM line (12).[4] This observation suggested that an alteration of the cell surface is involved in vinca alkaloid resistance. Using sodium [^{3}H]borohydride (NaB[^{3}H]$_4$) and galactose oxidase (GO) (17) to label the cell-surface glycoproteins (gp) of alkaloid-resistant leukemic cells, we were able to demonstrate two major changes: (*a*) the appearance of a high-molecular-weight gp of ≈ 180 kilodaltons (Kd), and (*b*) the concomitant disappearance of a lower-molecular-weight gp of ≈ 90 Kd (12). By comparison with cells of lower alkaloid resistance, it was possible to show a direct relationship between the degree of resistance, up to about 270-fold, and

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[4]We have recently been able to clone such morphologic variants from the drug-sensitive parent CEM cells, which are also sensitive to the cytotoxic effects of the vinca compounds. While these latter cells appear to have different cell-surface glycoproteins (gp) than do other drug-sensitive, normal morphology (single-cell) clones, they do not express the resistance-associated gp (Beck and Cirtain, unpublished results).

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TABLE 1.—Cross-resistance properties of CEM/VLB$_{100}$ cells

Drug	Degree of resistance or cross-resistance*
VLB	420
VCR	> 600
Vindesine	552
Daunorubicin	111
Colchicine	103
Etoposide	20
Podophyllotoxin	4.5
Methotrexate	2.9
6-Mercaptopurine	2.5

*Determined by dividing the IC_{50} for the resistant line (CEM/VLB$_{100}$) by the IC_{50} for the sensitive CEM cells. The IC_{50} values were those in which 48-hr cell growth was inhibited by 50%, compared to controls.

the amount of the high-molecular-weight protein (12). Similar results have been demonstrated by others in hamster cells resistant to colchicine, dactinomycin, daunorubicin, and VCR (18,19).

These cell-surface changes were confirmed and extended using the two-dimensional (2-D) analytic electrophoresis method of O'Farrell (20). As seen in figure 1, the appearance of a high-molecular-weight surface gp (arrow 1; gp180) and the loss of a lower-molecular-weight gp(s) (arrow 2; ≈ 90 Kd) are associated with alkaloid resistance. The areas designated "C" represent diffusely labeled gp of ≈ 110-130 Kd, common to both resistant and sensitive cells. At this time, it is still not known if the appearance of gp180 is related to the disappearance of gp90; also unclear is the role of either of these gp in natural product resistance. Antibodies directed against one or both of these proteins should help to resolve this question. Their apparent microheterogeneity is a common observation with such surface molecules (21), and suggests that these gp exist in several forms, each with a different degree of glycosylation. Whether the different glycosylated forms are responsible for bringing different amounts of gp180 into the membrane remains unknown. Finally, with regard to this figure, the isoelectric point of gp180 appears to be ≈ 5.5, whereas for a similar protein from hamster cells, it was recently reported to be ≈ 7.0 (19). Hence, there may be species differences in glycosylation of this resistance-associated protein.

Fluorograms of 2-D broad-range O'Farrell gels (22) of total cellular proteins labeled with [^{35}S]methionine are shown in figure 2. While gp180 is not detectable (most likely because of a slow turnover and/or low methionine content of this molecule), few or no differences between sensitive and resistant cells are seen elsewhere, except at arrow p30, which indicates a cytoplasmic protein of ≈ 30 Kd, pI ≈ 5.2 that appears to be present in greater amounts in the resistant cells. The association with and role of p30 in alkaloid resistance is unknown at this time, but it is of interest that a cytoplasmic protein of ≈ 19 Kd, pI ≈ 5.7, believed to be associated with resistance to VCR in murine cells, has been described by Meyers and Biedler (23).

Proteins tentatively identified as tubulins ("T" in fig 2), based on their comigration in two dimensions with purified rat brain tubulin, may also be altered in alkaloid-resistant cells. Figure 2, a broad-range gel, does not resolve tubulin subunits well; for this purpose, narrow-range 2-D gels are required. Definite assignment of these proteins as tubulins, however, will depend on results of "Western" blot experiments (24) and reactions with antitubulin antibody, currently in progress.

That there may be more than one biochemical lesion associated with alkaloid resistance is consistent with results of studies of alkaloid- and antibiotic-resistant hamster cells from Biedler's laboratory (19,25). Whether cytoplasmic changes in CEM/VLB$_{100}$ cells occur in concert with the surface gp changes or as separate steps in the selection process remains to be determined. One way to resolve this issue would be to double clone membrane-altered CEM/VLB$_{100}$ cells. Another would be to clone the gene coding for the high-molecular-weight resistance-associated gp, insert it into wild-type cells, and determine if other changes such as the appearance of p30, the disappearance of gp90, or even altered gangliosides (25) occur. Debenham et al (26) have apparently transferred DNA containing the gene(s) for multiple-drug resistance into drug-sensitive cells. To the best of my knowledge, however, they have not looked for expression of other resistance-associated proteins in these transfected cells. Finally, it might be possible to determine the relationship of multiple molecular changes to multiple-drug resistance by isolating mutants having high resistance to natural products but not expressing the resistance-associated gp. Conversely, it might also be possible to isolate drug-sensitive cells that do express this gp.

Pharmacologic Determinants of the Alkaloid-Resistant Phenotype

Many studies of the pharmacology of "natural products" in drug-resistant mouse and hamster cells have demonstrated decreased accumulation of drug, compared to the sensitive cells (7,27-33). Drug accumulation is likewise diminished in our alkaloid-resistant human leukemic cells. Figure 3 is a composite of two types of experiments, both employing standard silicone oil methodology for drug uptake and accumulation (34). Results between experiments tend to vary quantitatively, but not qualitatively. In panel A, uptake of [^{3}H]VLB over the first minute was measured by performing the assay directly in the microcentrifuge tubes; in panel B, drug accumulation approaching steady-state by 60 minutes is shown. Details of the methods, measurements of cell volumes, intercellular water-space, identity of the label, etc, have been de-

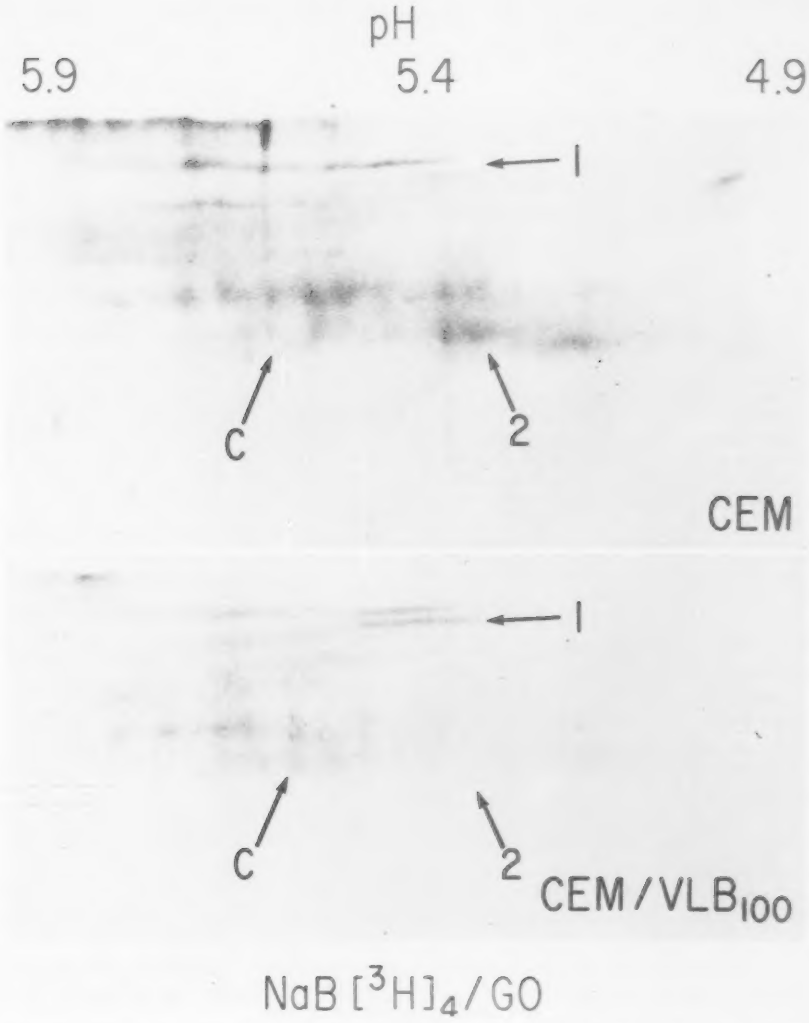

FIGURE 1.—2-D gel-analysis of cell-surface gp of VLB-sensitive (CEM) and -resistant (CEM/VLB_{100}) lymphoblasts. Surface labeling of cell gp was done with $NaB[^3H]_4$ and GO as described (ref 17). 2-D gel analysis was done according to the method of O'Farrell (ref 20) and subsequent fluorography was as described (ref 12). Arrows: 1 = gp180; 2 = gp90; C = gp of ≈ 110–130 Kd.

scribed (14). Two points are clear: (*a*) the initial uptake and subsequent accumulation of [^{3}H]VLB is reduced in resistant compared to sensitive cells; and (*b*) the high "zero-time" values indicate that there is a very large component of "instantaneous" binding by both cell lines. When calculated on the basis of intracellular water content, the concentration of VLB bound "instantaneously" is ≈ 10–20 times greater than the concentration of drug found in the medium, again suggesting involvement of a binding process. The high amount of binding to either intracellular or surface components greatly complicates determination of the actual mechanism of transmembrane transport of VLB.

Drug Retention in Alkaloid-Resistant Human Leukemia Cells

Decreased drug accumulation in alkaloid- and antibiotic-resistant mouse and hamster cells has been ascribed to

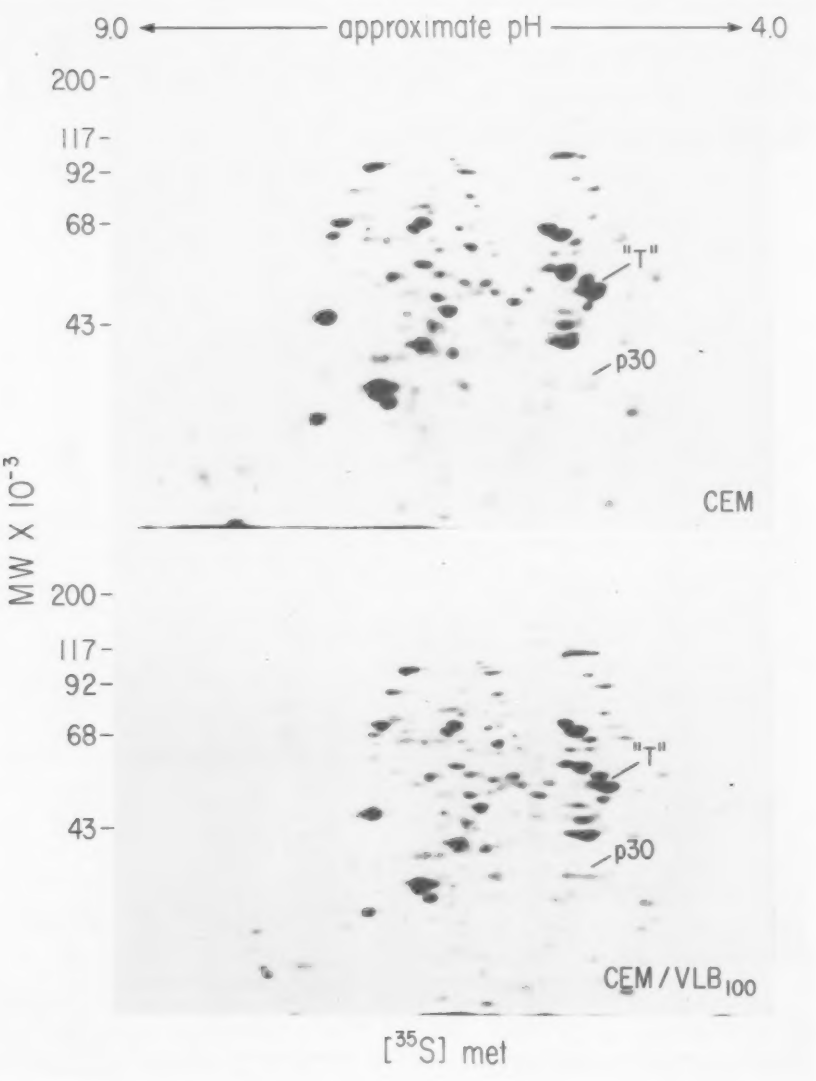

FIGURE 2.—Broad-range 2-D gel-analysis of [^{35}S]-methionine-labeled VLB-sensitive (CEM) and -resistant (CEM/VLB$_{100}$) cells. Cells were labeled with [^{35}S]methionine for ≈ 24 hrs in minimal essential medium (containing 10% fetal bovine serum), after which they were harvested by centrifugation, washed, and the pellet dissolved in O'Farrell sample buffer (ref 20). Broad-range 2-D gels were run as described (ref 22), and fluorography was as indicated earlier (ref 12). Arrows: p30 = cytoplasmic protein, ≈ 30 Kd, pI ≈ 5.7; "T" = apparent tubulins.

a decreased cellular capacity to retain the drug (5,30,31,35,36). The same appears true for our alkaloid-resistant human leukemic lymphoblasts. When preloaded with approximate equitoxic concentrations[5] of [^{3}H]VLB to steady-state levels, washed, and resuspended in drug-free medium, the cells lost drug until only presumably tightly bound [^{3}H]VLB remained (28,37). It can be seen in figure 4 that whereas > 40% of the drug associated with the sensitive cells was tightly bound and nonexchangeable, this fraction represented only about 10% of the total

[5]The IC$_{50}$ for CEM and CEM/VLB$_{100}$ cells used in *these* experiments were ≈ 3 and 330 nM, respectively.

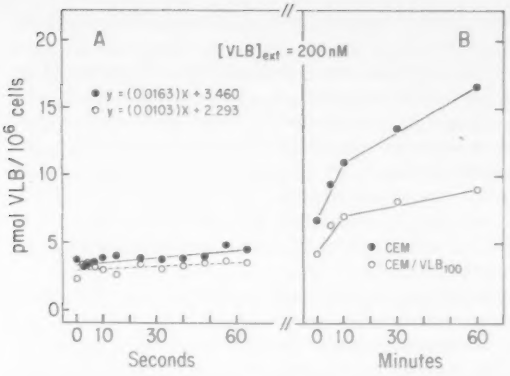

FIGURE 3.—Accumulation of [^{3}H]VLB by CEM and CEM/VLB$_{100}$ cells. Using standard silicone oil methodology (ref 34), drug accumulation at 37°C was examined over the 1st min (panel A) and over the 1st hr (panel B). Incubation buffers and conditions were as described (ref 14). Incubations in panel A were done directly in microcentrifuge tubes, and those in panel B were done in vials. External concentration of [^{3}H]VLB was 200 nM. Aliquots were taken for the determination of cell-associated ^{3}H at the times indicated. Regression lines for initial accumulation were fit by the least-squares method according to the equations shown in the fig.

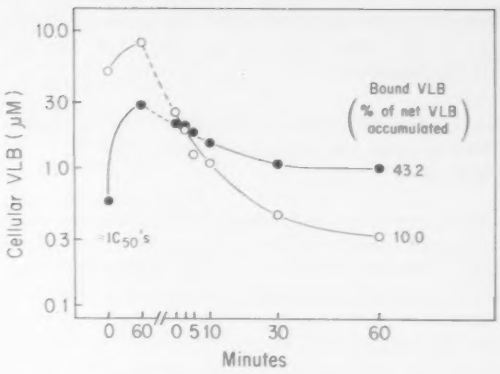

FIGURE 4.—Retention of [^{3}H]VLB by CEM and CEM/VLB$_{100}$ cells. Cells were incubated at 37°C in buffer containing [^{3}H]VLB at approximate equitoxic concentrations, 3 nM for CEM and 330 nM for CEM/VLB$_{100}$. At 60 mins, the cells were washed at 4°C and resuspended in drug-free buffer at 37°C. Aliquots of the cell suspension were taken for the determination of cell-associated ^{3}H at the times indicated. Shown is a representative experiment. Details of the method were as described (ref 14).

VLB accumulated by the CEM/VLB$_{100}$ cells. This was equally true for sensitive and resistant cells incubated in equal concentrations of VLB (200 nM) (14). Thus, the total releasable drug was greater in the resistant cells than in the sensitive cells.

Alkaloid Resistance and Efflux "Pumps"

It is clear that alkaloid-resistant human leukemic cells have a decreased ability to retain drug, which may account in part for the observed drug insensitivity. Whether this diminished retention stems from enhanced drug efflux or reduced drug binding has not been resolved. The former concept is favored as the explanation for resistance to natural products (29–31, 38–41), whereas the latter has been suggested in only a few cases (14,35,42,43).

The evidence for enhanced drug efflux comes primarily from studies demonstrating that metabolic inhibitors cause an increase in the steady-state level of drugs; addition of an energy source such as glucose to these inhibited cells leads to a rapid loss of drug to a new lower steady-state level (29–31,38–41). The interpretation of such results almost always entails an "active efflux drug pump" that has been poisoned, leading to enhanced drug accumulation. The "poisoned" efflux pump is thought to be activated by the new energy source, so that drug is extruded from the cell.

While this "pump" concept is attractive, it raises several questions that must be considered. First, if such a pump exists, what is its natural substrate(s)? Other than steroids, which apparently traverse cell membranes by unmediated simple diffusion (44), no other large, heterocyclic molecules of mammalian origin come to mind. Second, how can a pump have such "broad specificity" as to recognize molecules with vastly different structures, eg, vinca alkaloids, anthracyclines, and epipodophyllotoxins (30,40,41,45)? The inference from experiments with inhibitors is that the pump is a protein or group of proteins capable of actively transporting these molecules out of the cell. Third, does drug in fact exit cells by an active pumping mechanism? By definition, an active pump would require ATP, move a substrate against an electrochemical gradient, exhibit substrate saturability, temperature sensitivity competition by structural analogs, and be coupled to the transport of other substances, such as ions (37). To the best of my knowledge, natural product "efflux" has not been examined rigorously for most of these features. One exception, however, is that compounds for which cross-resistance has been demonstrated (anthracyclines, epipodophyllotoxins, and colchicine; table 1) do not prevent the glucose-mediated release of [^{3}H]VLB (14).

My working hypothesis is that alkaloid-sensitive and -resistant cells bind drugs differently, and that the greater fraction of "releasable" VLB in resistant cells most likely results from differences in binding. I suggest that cellular metabolism, rather than activating an efflux pump, may mediate changes in cellular drug-binding sites in resistant cells, possibly by causing their phosphorylation and hence altering their conformation to one that is unfavorable for binding. Moreover, such binding need not require the same structural specificity demanded by an efflux pump, but rather may only require differences in net charge (as would be expected of a phosphorylated

protein) to cause the release of several classes of agents, such as the vinca alkaloids, anthracyclines, and epipodophyllotoxins.

What Is the Relationship Between Altered Cellular Pharmacology and Resistance-Associated Molecules?

Since decreased drug accumulation by alkaloid-resistant human leukemic cells appears largely due to their reduced ability to retain the drug, it is important to identify the protein(s) most closely related to this deficiency, regardless of the actual mechanism of decreased retention. Several candidate proteins or classes of proteins are as follows: 180 Kd gp (gp180), 90 Kd gp (gp90), 30 Kd protein (p30), tubulins, Ca^{++}-Mg^{++} ATPases, calmodulin, and glycocalyx. The most obvious is the high-molecular-weight resistance-associated gp (180), yet several lines of evidence suggest that its relationship to drug resistance is complex. For example, experiments with tunicamycin (TM), a specific inhibitor of protein glycosylation (46,47), indicate that the carbohydrate portion of gp180 may not be necessary for the expression of resistance. We showed (13) that while TM treatment of CEM/VLB_{100} cells prevented the glycosylation of both gp180 and gp90, it affected neither the net steady-state accumulation nor the retention of VLB, nor did it appear to potentiate the drug's cytotoxicity. Our conclusion is that the carbohydrate component of gp180 is not required for expression of alkaloid resistance by these human leukemic lymphoblasts. Similarly, Chou and Kessel (48) treated doxorubicin-resistant P388/ADR cells with TM and found no effect of this agent on anthracycline resistance.

The results of these TM experiments should be interpreted with some caution, however. First, TM has been shown to have little or no effect on the functions of many gp (49,50), indicating that carbohydrates in general do not govern protein function or enzyme activity. Hence, this pharmacologic approach may not yield sufficient detail about the relationship of oligosaccharide to apoprotein. Second, while we could not demonstrate by one-dimensional gel analysis any effect of TM on the *types* of cellular proteins made (13), overall protein synthesis was depressed by this agent (Beck and Cirtain, unpublished results), suggesting that we may not have been able to detect a subtle effect of TM on the apoprotein of gp180. Use of 2-D gel methods and/or anti-gp180 antibodies should help to resolve this question.

Debenham et al (26) performed elegant "transfection" experiments showing that DNA-mediated transfer of colchicine resistance and natural product cross-resistance was associated with the expression of a resistance-associated gp ("P"-protein). Yet, the results of their studies with a "specific" antiserum and Western blot methods suggest that genes coding for other proteins were transferred as well, and might also be associated with resistance. (Indeed, it would be surprising if DNA coding for other proteins were *not* transfected along with the gene for the P-protein. Another possibility is that several resistance-associated proteins, including the high-molecular-weight gp, are coded for by linked genes or a family of genes, all under the control of a common regulator and capable of being transfected together.) Hence, while gp180 remains the favored candidate for a natural product "binding" protein, its relationship with altered drug binding and decreased drug effectiveness remains to be established.

As mentioned earlier, the appearance of gp180 on resistant leukemic blasts coincides with the disappearance of a lower-molecular-weight gp (gp90) (12). Similar results have been demonstrated by others (17,21,51) in Chinese hamster cells. We also demonstrated that TM blocks glycosylation of this gp (13). Although we have speculated that the amounts of gp90 and gp180 may be inversely related with regard to resistance (12), and Garman and Center (51) followed the disappearance of the lower-molecular-weight surface gp with development of anthracycline resistance, no direct relationship between these reciprocal changes has yet been established. One attractive hypothesis is that diminished drug retention is due to loss of the low-molecular-weight gp(s), rather than gain of the high-molecular-weight gp, and that gp90 accounts for alkaloid binding.

Tubulins are clearly proteins that, if altered in amount, affinity for alkaloids, subcellular distribution, turnover, or state of phosphorylation, could mediate the diminished drug accumulation and retention seen in alkaloid-resistant cells. While altered tubulins have been demonstrated in some alkaloid-resistant Chinese hamster ovary cells (52-54), it is not clear whether other resistance-associated changes (eg, the high-molecular-weight surface gp) were present in these cells. We are presently examining the tubulins of our membrane-altered CEM/VLB_{100} cells for such changes.

The low-molecular-weight, acidic protein demonstrated in the cytosols of VCR-resistant mouse and hamster cells, but not those of dactinomycin-resistant cells (23), may play a role in alkaloid resistance by mediating diminished drug accumulation. This protein ($M_r \approx 19{,}000$; pI, 5.7) is expressed in cells that also possess the high-molecular-weight ($M_r \approx 150{,}000$) gp (19). Whether the similar cytosolic protein shown in figure 2 (p30) also plays a role in diminished alkaloid accumulation is not clear.

The interesting work of Tsuruo et al (55-57), demonstrating that calcium antagonists and calmodulin inhibitors can make alkaloids and anthracyclines more effective against drug-resistant cells, suggests that calcium metabolism may be involved in resistance to the vinca alkaloids or anthracyclines. Accordingly, proteins such as Ca^{++}-Mg^{++} ATPase(s) and/or calmodulin are potential targets for investigation in resistance to these anticancer drugs. It should be noted, however, that the work of

Tsuruo has only indirectly implicated calcium. Since neither the levels, fluxes, nor subcellular distribution of calcium have been measured in resistant and sensitive cells, any role for calcium in alkaloid resistance must remain for the present time speculative. Moreover, calmodulin levels in our VLB-resistant human leukemic cells did not differ from levels in the drug-sensitive CEM cells (Beck and Tallant, unpublished results).

Finally, studies of Minor and Roscoe (35) distinguished apparent colchicine-binding compartments that differed between colchicine-sensitive and -resistant hamster cells. This suggested that one of the compartments responsible for loose binding of drugs by the resistant cells may be associated with the glycocalyx. Our experiments likewise permit identification of two different binding components in resistant and sensitive lymphoblasts (14). Insofar as the glycocalyx binds most of the cell's calcium (58), it becomes an attractive target for study.

The biochemical and pharmacologic alterations found to date in alkaloid-resistant cultured human leukemic lymphoblasts are summarized in table 2. Several of these alterations have been shown to be associated with the multiple-drug resistance phenotype in mouse and hamster cell lines, indicating common features for drug-resistant rodent and human cell lines. Future investigations will focus on the identification of other resistance-associated proteins and will attempt to determine the relationship between vinca alkaloid resistance and these molecular changes in the cell. An understanding of the molecular mechanism of multiple-drug resistance should provide a rational basis for probing tumor cell populations for drug-resistant subpopulations.

TABLE 2.—Biochemical and pharmacologic expressions of vinca alkaloid resistance in CCRF-CEM cells

Molecular alterations*
↑ gp180
↓ gp 90
↑ p 30
Tubulins?
Pharmacologic alterations*
↓ Drug accumulation
↓ Drug retention
Large bound fraction
Energy-dependent drug binding
↓ Cytotoxicity of alkaloids and other natural product drugs

* Arrows indicate increase (↑) or decrease (↓).

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Evidence for Multidrug-Resistant Cells in Human Tumor Cell Populations[1]

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Data are presented from two cell culture systems which support the notion that a multidrug-resistant phenotype occurs in human tumor cell populations. Human small cell lung cancer cell lines derived from patients in relapse following intensive combination chemotherapy demonstrate broad cross-resistance to nine standard drugs in vitro. However, analysis of [^{14}C]glucosamine-labeled glycoproteins in the small cell lung cancer cell lines failed to identify any consistent association between a specific glycoprotein marker and the drug-resistant phenotype. Evaluation of drug sensitivity of human tumor cells in primary culture (colony-forming assay) has indicated that multidrug-resistant cells may be present in tumor cell populations even in the absence of prior drug therapy. Several features of the multidrug-resistant phenotype, as observed in these human tumor cell populations, differ from those observed in Chinese hamster cell systems. In particular, the variability in patterns of resistance to various agents and in expression of glycoprotein markers suggests that a substantial amount of genetic heterogeneity underlies this phenotype in human tumors. [Cancer Treat Rep 67:883–888, 1983]

A phenomenon of pleiotropic or multidrug resistance has been described in Chinese hamster (1,2) as well as murine (3–6) cell systems. As described elsewhere in this symposium, detailed studies in Chinese hamster lung and ovary cells selected in vitro for resistance to vincristine or colchicine have indicated that broad-spectrum resistance to multiple agents with different mechanisms of action is associated with reduced intracellular accumulation of drug and specific changes in membrane glycoproteins. Evaluation of a human lymphoblastic leukemia cell line selected for resistance to vinblastine (7) suggests that this phenomenon may also occur in human cells. The purpose of this paper is to present additional evidence for the occurrence of a multidrug-resistant phenotype in clinically derived human tumor cell populations and to compare the phenotype with that observed in the Chinese hamster systems.

In Vitro Drug Sensitivity and Glycoprotein Characteristics of Human Small Cell Lung Cancer Cell Lines

Methods for establishment and characterization of human small cell lung cancer (SCLC) cell lines have been previously described (8,9). Of importance to the present study are the presence of human isozymes and karyotype, the presence of APUD markers, and the expression of tumorigenicity following implantation of the cell lines in nude mice. In vitro assessment of drug sensitivity was performed using a soft-agarose cloning assay (8,10). Tumor cell lines were considered sensitive to a given agent where colony formation was reduced to $\leq 30\%$ of the control at $\leq 10\%$ of the achievable peak plasma concentration.

Agents tested included those with known clinical activity in the treatment of SCLC. In general, cell lines

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derived from patients with progressive disease during combination chemotherapy showed in vitro resistance to most agents tested, while cell lines from previously untreated patients were uniformly sensitive. It should be noted that this previously treated patient population had been exposed to intensive combination chemotherapy (six to eight drugs). A total of 40 drug assays were performed on cell lines derived from resistant, relapsed patients, and in only six instances were the tumor cells sensitive to a single agent. These data suggest that a pattern of pleiotropic drug resistance (PDR) occurs in SCLC. However, in contrast to reports of PDR in Chinese hamster cells, most SCLC cell lines were sensitive to colchicine.

Glycoprotein characteristics of the SCLC cell lines were analyzed after labeling with [^{14}C]glucosamine, solubilization in sodium dodecyl sulfate, and electrophoresis on polyacrylamide gels. Figure 1 presents an electrophoretogram for eight different cell lines from both newly diagnosed patients and patients in relapse receiving multiple-agent chemotherapy. No uniform glycoprotein "marker" for resistance was observed in the five cell lines derived from the patients in relapse. Overall, a heterogeneous pattern is observed, with each cell line demonstrating a unique glycoprotein "fingerprint" which bears no obvious relation to drug responsiveness.

Drug Sensitivity/Resistance of Human Solid Tumors in Primary Cell Culture

A data base comprised of > 6000 drug-sensitivity tests derived from evaluation of 566 previously untreated human tumors was examined for the occurrence of multidrug-resistant tumors. This data base was generated as part of an NCI contract program for application of a human tumor colony-forming assay to new drug screening (11). Drug-sensitivity testing was performed essentially as described by Hamburger and Salmon (12) using a two-layer soft-agar culture system. Drug exposure was by the "continuous" method. Drug solutions were combined with cell suspensions immediately prior to plating to achieve a final concentration of 10 μg/ml in the culture

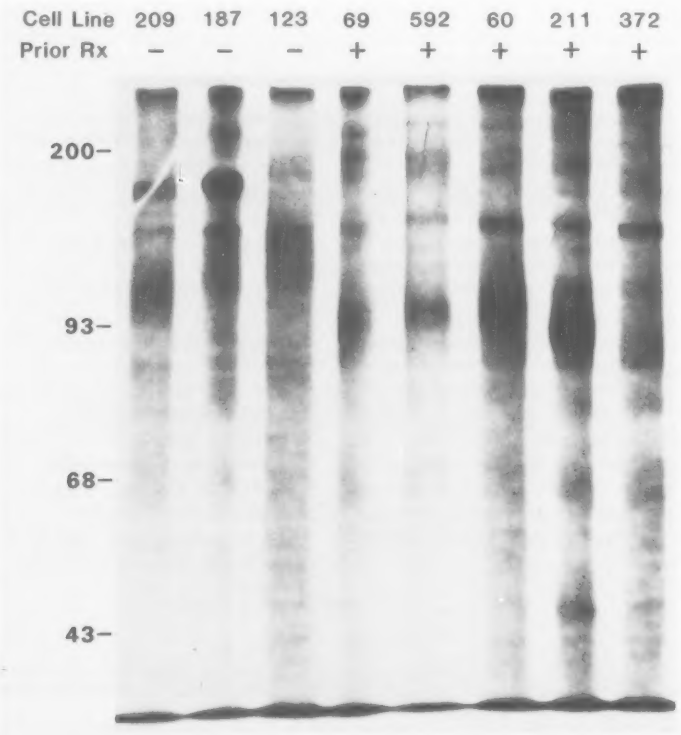

FIGURE 1.—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [^{14}C]glucosamine-labeled cell extracts of 8 human SCLC cell lines. Each cell line is listed with its history of prior therapy. Molecular weight standards, in thousands, are shown at the left border of the gel.

TABLE 1.—Activity ranking of known compounds in the human tumor clonogenic assay

Compound	Fraction of tumors sensitive (%)
Chromomycin A_3	15/17 (88.2)
Anguidine	23/37 (62.1)
Dactinomycin	15/30 (50.0)
Doxorubicin	16/33 (48.4)
Mitomycin	12/29 (41.3)
Maytansine	6/17 (35.2)
Mercuric chloride	10/29 (34.4)
Bleomycin	9/30 (30.0)
5-FU	9/32 (28.1)
Vinblastine	5/19 (26.3)
Cisplatin	7/27 (25.9)
Chlorambucil	5/31 (16.1)
Carmustine	5/36 (13.8)
Melphalan	3/28 (10.7)
Sodium chloride	2/34 (5.8)
L-Glutamine	1/28 (3.5)
D-Glucose	0/31 (0.0)
Sodium azide	0/30 (0.0)
D-Mannitol	0/26 (0.0)
Water	0/20 (0.0)

plates. Colony formation was quantitated after 7–21 days using a computerized image analysis system.

A large number of known compounds have been tested during efforts to establish the validity of this system for drug screening. Table 1 summarizes testing results obtained with the current protocol for 20 compounds including standard antitumor agents, toxic compounds without therapeutic efficacy, and nontoxic compounds. Tumors were considered sensitive if a given agent reduces colony formation to $\leq 30\%$ of the control colony number.

To evaluate this data base for the occurrence of tumors demonstrating a multidrug-resistant phenotype, an operational definition of the phenotype was established. Four compounds consistently associated with the multidrug-resistant phenotype in Chinese hamster cell populations were selected to establish a test battery. These included dactinomycin, doxorubicin, vinblastine, and colchicine. In this operational definition of a multidrug-resistant phenotype, tumors were considered to manifest the phenotype if they demonstrated resistance to three or more of the compounds. Table 2 summarizes the data on tumors resistant to individual drugs included in the test battery. A significant frequency of resistance was observed for each of these agents. Data on tumors resistant to two of these agents are presented in table 3. A somewhat smaller but still significant number of tumors were observed to show resistance to various pairs of drugs. The highest degree of cross-resistance was noted between agents with similar modes of action, eg, vinblastine and colchicine. Figure 2 illustrates the drug-sensitivity profile of a breast tumor in this category. In addition to resistance to doxorubicin and colchicine, this tumor was resistant to a number of standard anticancer agents including carmustine, bleomycin, cisplatin, and 5-FU. Table 4 presents the results obtained when the data base was examined for the occurrence of tumors resistant to three of the agents included in the battery. It can be seen that a rather small number of tumors were actually tested against three of these agents. Even so, resistance to three agents was a relatively uncommon event. Figure 3 illustrates the drug-sensitivity profile of an adrenal tumor in this category. In addition to dactinomycin, colchicine, and vinblastine, this tumor was resistant to cisplatin and melphalan. Among the tumors sensitive to multiple agents, resistance to other agents was also observed. Figure 4 illustrates the drug-sensitivity profile of a colorectal tumor. While this tumor was quite sensitive to two components of the test battery (dactinomycin and doxorubicin) and to bleomycin, it was relatively resistant to melphalan and mitomycin.

In attempting to apply this operational definition of a multidrug-resistant phenotype to the data base retrospectively, it becomes obvious that while tumors fitting the definition are observed, this arbitrary and rather simplistic approach is not fully descriptive of the drug-resistance phenomena observed in these tumors. The data available on compounds other than those included in the operational definition contribute important information. Overall, while tumors may be resistant to a number of different agents, the patterns of cross-resistance are heterogeneous. Resistance to any one drug

TABLE 2.—Tumors resistant to individual drugs in vitro

Drug	Fraction of tumors resistant (%)
Dactinomycin	32/65 (49)
Doxorubicin	82/124 (66)
Vinblastine	51/68 (75)
Colchicine	29/38 (76)

TABLE 3.—Tumors resistant to 2 drugs in vitro

Drugs	Fraction of tumors resistant (%)
Dactinomycin and doxorubicin	11/33 (33)
Dactinomycin and vinblastine	2/17 (12)
Dactinomycin and colchicine	7/20 (35)
Doxorubicin and vinblastine	10/23 (44)
Doxorubicin and colchicine	3/7 (43)
Vinblastine and colchicine	6/10 (60)

TABLE 4.—Tumors resistant to 3 drugs in vitro

Drugs	Fraction of tumors resistant (%)
Dactinomycin, doxorubicin, and vinblastine	0/9 (0)
Dactinomycin, doxorubicin, and colchicine	1/5 (20)
Dactinomycin, vinblastine, and colchicine	1/7 (14)
Doxorubicin, vinblastine, and colchicine	1/3 (33)

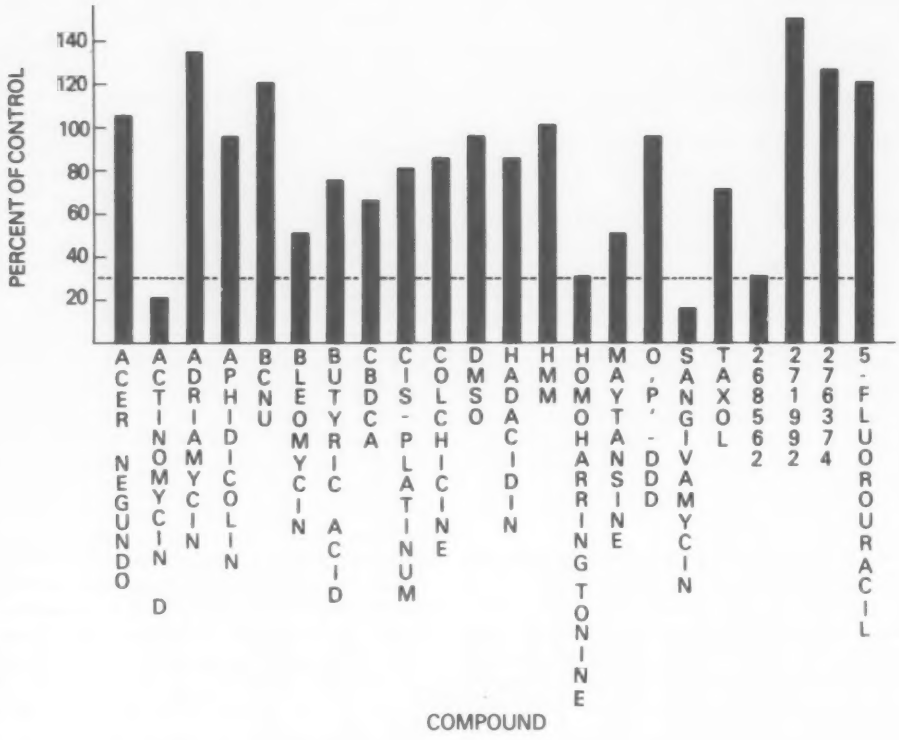

FIGURE 2.—Drug-sensitivity assay results for a breast tumor. For each agent, % survival values (No. of colonies in treated plates/No. of colonies in control plates) are shown as vertical bars. Using reduction of colony formation to ≤ 30% of the control value as the criterion for sensitivity, this tumor was resistant to doxorubicin and colchicine as well as several other agents.

does not appear to be closely linked to resistance to other agents with different mechanisms of action as has been demonstrated in in vitro systems.

DISCUSSION

We have presented data from two cell culture systems which indicate that a multidrug-resistant phenotype does occur in human tumor cell populations. (*a*) SCLC cell lines from patients resistant to clinical chemotherapy show broad-spectrum drug resistance in vitro. (*b*) A number of tumors from previously untreated patients show a heterogeneous pattern of resistance to multiple agents in primary cell culture. It would seem, however, that such a phenotype is not identical to that observed in the Chinese hamster cell systems. Table 5 presents a comparison of the phenotype as observed in the Chinese hamster cells (1,2), as observed in human leukemic lymphoblasts (7), and as reported here. The phenotype observed in human leu-

TABLE 5.—Comparison of multidrug-resistant phenotype in Chinese hamster and human cells

Drug resistance	Chinese hamster ovary cells	Chinese hamster lung cells	Human leukemic cell line (CCRF-CEM)	Human SCLC cell lines	Human tumor cells in primary culture
Anthracyclines	+	+	+	+	+
Vinca alkaloids	+	+	+	?	+
Colchicine	+	+	+	-	+
Other drugs	+	+	+	+	+
Membrane glycoprotein marker	+	+	+	?	?

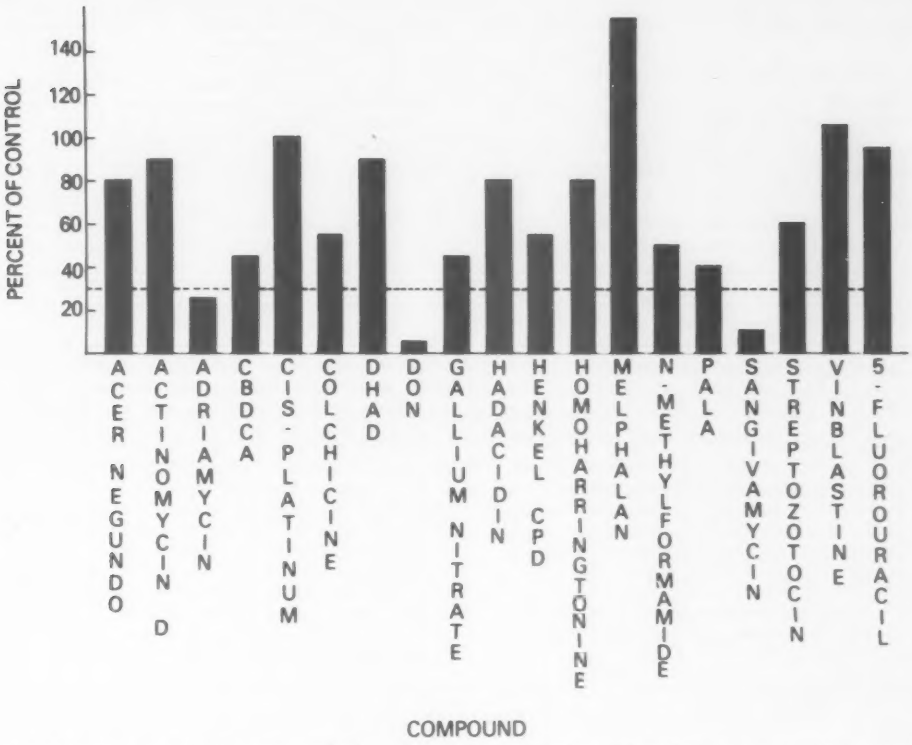

FIGURE 3.—Drug-sensitivity profile of an adrenal tumor resistant to dactinomycin, colchicine, and vinblastine. This tumor was also resistant to cisplatin, melphalan, and 5-FU. Data presentation and activity criteria are as described for fig 2.

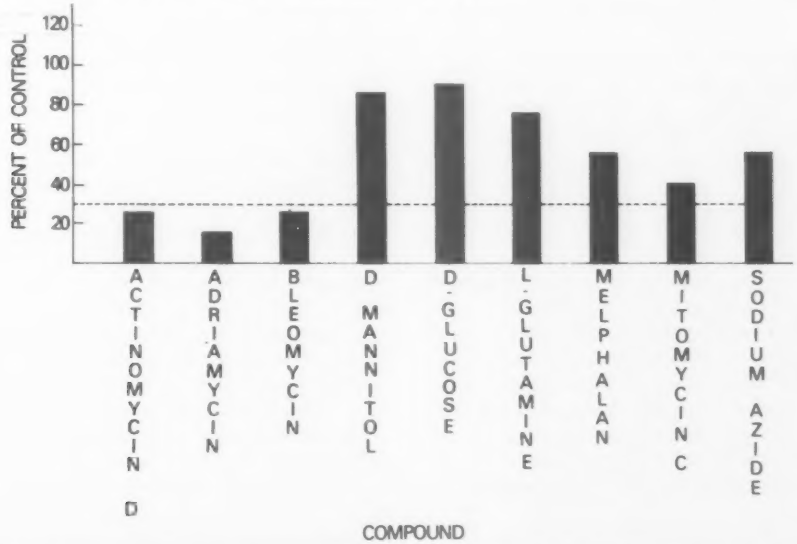

FIGURE 4.—Drug-sensitivity profile for a colorectal tumor. This tumor was sensitive to 2 agents included in the operational definition of multidrug resistance, but it was resistant to 2 other established agents. Data presentation and activity criteria are as described for fig 2.

kemic lymphoblasts selected in vitro for resistance to vinblastine very closely resembles that observed for Chinese hamster cells. Some differences appear to exist in the compounds involved in drug resistance and in the role which cellular glycoproteins may play in the phenomenon. SCLC cell lines derived from heavily pretreated patients demonstrated multidrug resistance in vitro, yet remained sensitive to colchicine. While human tumors tested in primary culture clearly may be resistant to any of the compounds which have been associated with PDR in hamsters, the pattern of association of these agents in a multidrug-resistant phenotype is highly heterogeneous. These observations support the notion that a substantial amount of genetic heterogeneity may underlie the multidrug-resistant phenotype in human tumor cell populations.

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Reversal of Acquired Resistance to Vinca Alkaloids and Anthracycline Antibiotics[1]

Takashi Tsuruo[2,*]

Reversal of acquired resistance to vinca alkaloids and anthracycline antibiotics has been attained in vitro and in vivo by some calcium influx blockers and calmodulin inhibitors. These calcium influx blockers and calmodulin inhibitors enhance the intracellular level of vincristine and doxorubicin in tumor cells, especially in drug-resistant mouse and human tumor cells, by inhibiting their outward transport. Among tumor cell lines established from mice and humans, less sensitive tumor cells became more susceptible to vincristine by use of calcium influx blocker, and inherent resistance of tumor cell lines to vincristine has also been circumvented. [Cancer Treat Rep 67:889–894, 1983]

One of the major causes of failure in cancer chemotherapy is the selection and proliferation of specific drug-resistant tumor cells during treatment (1,2). The mechanism of acquired resistance of tumor cells to some agents is related to intracellular drug accumulation and retention. For example, in vincristine (VCR)- and doxorubicin (ADM)-resistant tumor cell sublines, these agents can be shown to enter the cell but are actively transported to the outside (3–6). This results in a relatively low intracellular level of drug and thus to low cytotoxicity (3–6). These observations suggest that if we could control the VCR- and ADM-efflux function of resistant tumor cells appropriately, then we could expect a reversal of acquired resistance to these drugs in drug-resistant tumor cells.

We found that modifiers of cellular calcium modulate the cytotoxicity and cellular accumulation of vinca alkaloids (7,8). Recently, we found that verapamil, a calcium influx blocker, efficiently inhibits the VCR- and ADM-efflux function of tumor cells, especially of resistant tumor cells (6,9). VCR and ADM accumulate greatly in resistant tumor cells when verapamil is used, and the increased intracellular levels of these drugs are directly related to the reversal of resistance to these drugs (6,9). Subsequent studies revealed that calmodulin inhibitors possessed activity similar to that of calcium influx blockers (10,11). Inherent drug resistance of heterogeneous tumor cells (12) has also been circumvented by this approach (13,14). Potentiation of VCR and ADM effects has been achieved in human hematopoietic tumor cell lines, especially in resistant cells (14).

The present report describes our findings on the reversal of acquired resistance to vinca alkaloids and anthracycline antibiotics. The therapeutic advantages of this approach will be described briefly.

Enhancement of Cytotoxic Effects of VCR and ADM in P388, K562, and Their VCR- and ADM-Resistant Cells by Verapamil

Tumor cells were treated with VCR or ADM with verapamil; after continuous exposure to drug for 72 hours, the cells were counted and the IC50 values (drug concentrations required for 50% inhibition of cell growth) of VCR or ADM in the presence or absence of verapamil were determined.

P388/ADM cells (P388 cells resistant to ADM) were highly resistant to ADM (index of resistance [IR], 27) and also highly resistant to VCR (IR, 46.9) (table 1). P388/VCR cells (P388 cells resistant to VCR) were highly resistant to VCR (IR, 25.6); however, the resistance to ADM was rather low (IR, 3.4). Human myelogenous K562 leukemia resistant to VCR (K562/VCR), which was established in this laboratory by continuous VCR exposure in vitro, was also highly resistant to VCR (IR, 16.9). However, the cells were again weakly resistant to ADM (IR, 3.0). These results indicate that ADM has a rather weak cross-resistance to VCR in VCR-resistant tumor cells,

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suggesting the possibility of minor differences in the mechanisms of ADM and VCR resistance in tumor cells.

Verapamil, at nontoxic concentrations of 2.2 and 6.6 μM, enhanced the cytotoxicity of VCR approximately fourfold against P388 or K562 cells as shown by comparison of IC50 values. The enhancement of ADM cytotoxicity was less than twofold. Verapamil greatly enhanced the cytotoxicity of VCR in VCR- or ADM-resistant P388 or K562 cells, with a maximum of 40- to 70-fold enhancement of VCR cytotoxicity. Enhancement of ADM cytotoxicity in P388/VCR or P388/ADM cells was approximately tenfold, and that in K562/VCR cells was approximately fourfold to fivefold. Verapamil is more effective for reversal of VCR resistance than for reversal of ADM resistance. VCR and ADM resistance in tumor cells again seems to be slightly different with respect to their responses to verapamil. The resistance to VCR was completely overcome, since the IC50 values for VCR in resistant tumor cells in the presence of verapamil were lower than those (1.6 or 2.9 nM) for VCR in P388 or K562 nonresistant cells.

Cellular Accumulation and Retention of VCR and ADM and Effect of Verapamil

Uptake of [^{3}H]VCR into cultured P388 cells increased with time (fig 1). The amounts of VCR in P388/VCR or P388/ADM cells were much smaller. The mechanism of resistance could be explained by this phenomenon. Verapamil added to the culture at 6.6 μM greatly increased the amount of cellular VCR in P388, P388/VCR, and P388/ADM cells (6,9). In particular, an almost 15- to 20-fold accumulation of VCR occurred in verapamil-treated P388/VCR or P388/ADM cells during 1–3 hours of incubation (fig 1A).

The accumulation of [^{3}H]ADM in P388/VCR and P388/ADM cells was considerably lower than that found in P388 cells at 2 hours (fig 1B). Verapamil also enhanced the cellular accumulation of [^{3}H]ADM in resistant cells; an approximately twofold to 2.5-fold increase occurred in P388/VCR and P388/ADM cells. Verapamil, however, did not enhance the cellular uptake of [^{3}H]ADM in P388 cells.

Enhancement of VCR or ADM cytotoxicity in P388/VCR, P388/ADM, and P388 cells by verapamil and reversal of drug resistance could be explained by this phenomenon. In P388 cells, the cellular accumulation and cytotoxicity of ADM were not enhanced significantly by verapamil, although verapamil evidently enhanced the effect of VCR in P388 cells.

The efflux of intracellular VCR from P388/VCR cells, or ADM from P388/ADM cells, was efficiently inhibited by verapamil (fig 1). These results indicate that the higher accumulation of VCR or ADM in drug-resistant tumor cells could occur through an inhibition of the efflux mechanism of these antitumor agents by verapamil.

Enhancement of Cytotoxicity of VCR and ADM in Drug-Resistant Tumor Cells by Calmodulin Inhibitors

Calmodulin inhibitors enhanced the cytotoxicity of VCR in P388/VCR and K562/VCR cells and the cytotoxicity of ADM in P388/ADM cells (10). Prenylamine enhanced VCR cytotoxicity up to 23-fold in P388/VCR cells, as compared with the IC50 values, and up to 45-fold in K562/VCR cells (table 2). Enhancement of ADM cytotoxicity was smaller than that observed for VCR, and a maximum of 11-fold enhancement of ADM cytotoxicity occurred with prenylamine. VCR resistance was circumvented by these modifiers in VCR-resistant tumor cells, since the IC50 values shifted to values lower than the IC50s for the parent cells; ADM resistance was partially overcome by these modifiers.

These modifiers at 6.6 μM enhanced cellular uptake of VCR and ADM by twofold to fivefold and approximately twofold, respectively (10). Since the outward efflux of the preloaded VCR or ADM was inhibited by the modifiers (10), the increase in intracellular accumulation of VCR and ADM by these calmodulin inhibitors seems at least partly due to the inhibition of extracellular transport of these antitumor agents (10). These results might indicate the possibility that calmodulin is involved in outward drug-transport mechanisms of resistant tumor cells, although the mechanism is not known at present.

TABLE 1.—Enchancement of VCR- and ADM-induced cytotoxicity by verapamil in P388, K562, and their drug-resistant cells*

Verapamil (μM)	P388	P388/VCR	P388/ADM	K562	K562/VCR
			IC50 (nM) of VCR		
0	1.6	41	75	2.9	49
2.2	0.55 (2.9)	1.3 (32)	5.4 (14)	1.8 (1.6)	3.8 (13)
6.6	0.42 (3.8)	1.0 (41)	1.1 (68)	0.72 (4.0)	0.8 (61)
			IC50 (nM) of ADM		
0	31	105	850	24	72
2.2	20 (1.6)	20 (5.3)	110 (7.7)	15 (1.6)	21 (3.4)
6.6	18 (1.7)	11 (9.5)	90 (9.4)	13 (1.8)	16 (4.5)

*Tumor cells were treated with VCR or ADM, with or without verapamil; after 72 hrs of continuous drug exposure the cells were counted. Cytotoxicity of VCR or ADM in the presence or absence of verapamil was measured by determining the IC50 values (drug concentration required for 50% inhibition of cell growth) (6,9,14). Values in parentheses = decrease (x-fold) in the IC50 value as compared to the IC50 without verapamil.

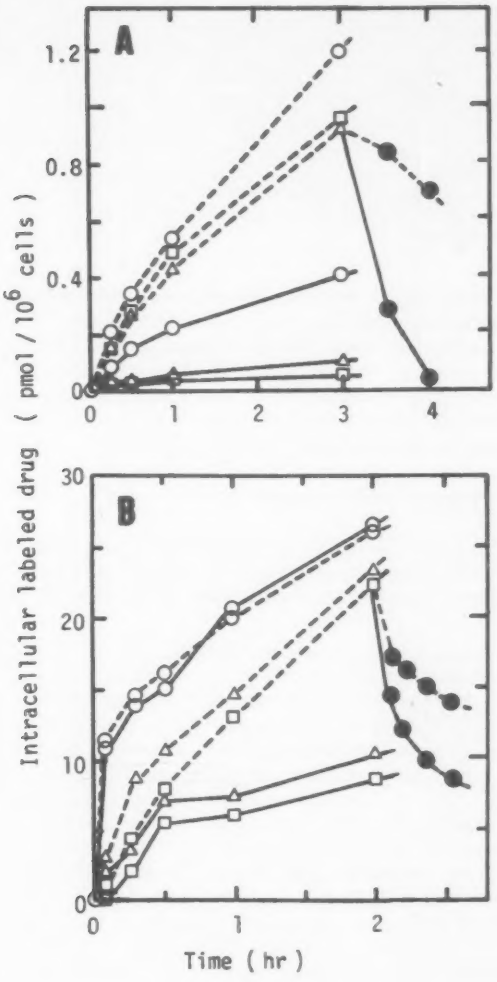

FIGURE 1.—Effects of verapamil on the intracellular and extracellular transports of [^{3}H]VCR or [^{3}H]ADM. P388 (○), P388/VCR (△), and P388/ADM (□) cells (each 2 × 10^5/ml of medium) were incubated at 37°C in growth medium with [^{3}H]VCR (33 nM; specific activity, 6.3 Ci/mmol) (panel A) or [^{3}H]ADM (200 nM; specific activity, 76 mCi/mmol (panel B) in the absence (———) or presence (- - -) of 6.6 μM verapamil. At time intervals, the intracellular accumulation of VCR (panel A) and ADM (panel B) was determined as described previously (6,9,10). At the end of incubation, P388/VCR cells loaded with [^{3}H]VCR in the presence of verapamil (panel A) or P388/ADM cells loaded with [^{3}H]ADM in the presence of verapamil (panel B) were centrifuged, and precipitated cells were incubated at 37°C in growth medium in the absence (———•———) or presence (- - -•- - -) of verapamil. At the time intervals indicated, the intracellular [^{3}H]VCR or [^{3}H]ADM levels were determined as described previously (6,10).

Potentiation of Antitumor Activities of VCR and ADM in Mice Bearing Drug-Resistant Tumor Cells

Calcium influx blockers and calmodulin inhibitor are effective against drug resistance in vivo (6,11). VCR given daily at 100 μg/kg for 10 days starting on Day 1 showed no therapeutic effect against P388/VCR-bearing mice. However, verapamil given ten times with VCR significantly increased the lifespan of the P388/VCR bearer (table 3). An increase in lifespan of approximately 5 days was observed at a verapamil dose of 100 mg/kg. Similar enhancement of VCR effect also occurred with other calcium antagonists, diltiazem, nicardipine, niludipine, and also with the calmodulin inhibitor clomipramine. Especially notable was an increase in lifespan of 4–6 days which occurred with diltiazem, nicardipine, and niludipine. These modifiers also enhanced the chemotherapeutic effect of ADM in mice bearing ADM-resistant tumor cells, although the effect was not as prominent as that observed for VCR treatment. An increase in lifespan of approximately 3 days occurred with verapamil, diltiazem, nicardipine, and niludipine.

Circumvention of the Heterogeneity in Drug Sensitivity

The heterogeneous nature of human tumor cells in their response to chemotherapeutic agents is the crucial problem in present cancer chemotherapy, as has been observed in animal experiments (1,2). Different tumor cells, isolated from a single tumor or from different tumors, usually showed inherently different sensitivities to antitumor agents (12). Sometimes, tumors do not respond well to chemotherapeutic agents, and in most cases, although a response is obtained, it is very difficult to attain a "cure" with regimens of only chemotherapeutic agents.

We have found that the present approach utilizing calcium influx blockers can potentiate responsiveness to the drug in tumor cell lines inherently resistant to it and can also circumvent the heterogeneity in drug sensitivity (13,14). Cultured cell lines LL, B16, C26, and C38 established from Lewis lung carcinoma, B16 melanoma, and colon adenocarcinomas 26 and 38, respectively, showed inherently different resistance to VCR in vitro (fig 2A). The C26 cell line was the most resistant and the B16 cell line was the most susceptible to VCR. Verapamil enhanced the cytotoxicity of VCR, depending on the susceptibility of cells to VCR. C26 cells became the most susceptible to VCR with a nontoxic concentration of verapamil. VCR cytotoxicity against each cell line reached almost the same level by use of verapamil. Thus, the inherent resistance to VCR among the tumor lines was circumvented.

The same phenomenon was also observed for cultured cell lines established from a single tumor (B16 melanoma). B16-F1, F10, B2, and BL-6 were metastatic variants

TABLE 2.—Enhancement of cytotoxicity of VCR and ADM by calmodulin inhibitors*

Calmodulin inhibitor	IC50 (nM) of VCR in P388/VCR cells	IC50 (nM) of ADM in P388/ADM cells	IC50 (nM) of VCR in K562/VCR cells
Prenylamine	1.0 (23)	82 (11)	1.1 (45)
Trifluoperazine	2.3 (10)	200 (4.5)	2.0 (25)
Clomipramine	2.0 (12)	250 (3.6)	3.5 (14)
No. 233	7.9 (2.9)	660 (1.4)	10.6 (4.6)
Control (without modifier)	23	900	49

*IC50 value of VCR or ADM was determined as described in table 1. Calmodulin inhibitors were used at the non-toxic concentration of 6.6 μM except for trifluoperazine which was used at 3.3 μM (10). Values in parentheses = decrease (x-fold) in the IC50 value as compared to the IC50 in control experiment.

established by Fidler (15). These cell lines, including parent B16 cells, showed different susceptibility to VCR (fig 2B). Verapamil circumvented the heterogeneity in VCR sensitivity among these tumor cell lines, and all tumor cells became very susceptible to VCR by use of verapamil.

Heterogeneity in VCR sensitivity among human hematopoietic tumor cell lines established from acute leukemia and lymphoma was also circumvented by verapamil (fig 2C). BALL and Daudi cells (B-cell type) respond well to VCR. The less sensitive tumor cell lines, including VCR-resistant K562 cells, became more susceptible to VCR by use of verapamil. The human tumor lines used in this experiment were established from different patients and do not necessarily represent the heterogeneity of the hematopoietic tumor cells of one patient. However, these results might suggest the possibility that less sensitive populations of hematopoietic tumor cells from one patient could be rendered sensitive to VCR, which could possibly lead to a higher "cure rate" clinically.

Therapeutic Advantages of the Approach

In summary, the advantages of the present approach are the following: (*a*) reversal of acquired resistance to vinca alkaloids and anthracycline antibiotics; (*b*) po-

TABLE 3.—Effect of calcium antagonists and calmodulin inhibitor on antitumor activity of VCR in P388/VCR- or P388/ADM-bearing mice*

Drug (dose)	Mean survival time (days) ± SD	T/C
Experiment 1		
Control	11.0 ± 0.6	100
VCR (100 μg/kg)	10.6 ± 0.5	96
+ verapamil (100 mg/kg)	16.0 ± 0	145†
+ verapamil (75 mg/kg)	15.0 ± 0.7	136†
Experiment 2		
Control	11.4 ± 1.2	100
VCR (100 μg/kg)	10.8 ± 1.3	95
+ diltiazem (125 mg/kg)	15.0 + 1.5	132†
+ diltiazem (100 mg/kg)	15.0 ± 0.9	132†
+ nicardipine (75 mg/kg)	17.2 ± 1.9	151†
+ nicardipine (60 mg/kg)	15.6 ± 1.0	137†
+ niludipine (75 mg/kg)	17.1 ± 1.7	150†
+ niludipine (60 mg/kg)	16.9 ± 1.5	148†
+ clomipramine (30 mg/kg)	14.4 ± 1.8	131†
+ clomipramine (20 mg/kg)	14.4 ± 2.0	131†
Experiment 3		
Control	12.6 ± 0.5	100
ADM (1.0 mg/kg)	14.8 ± 0.7	117
+ verapamil (75 mg/kg)	17.3 ± 2.0	137†
+ verapamil (50 mg/kg)	17.4 ± 1.4	138†
+ diltiazem (125 mg/kg)	17.6 ± 1.7	140†
+ nicardipine (75 mg/kg)	18.0 ± 2.0	143†
+ niludipine (75 mg/kg)	17.4 ± 1.7	133†
+ clomipramine (20 mg/kg)	13.6 ± 1.2	108

*Each group of 5 $CD2F_1$ mice was given intraperitoneal implants of 10^6 P388/VCR leukemia cells (Experiments 1 and 2) or 10^5 P388/ADM leukemia cells (Experiment 3), and drugs were given ip daily from Day 1 to Day 10 (6,11).
†Significant ($P < 0.05$) by t test as compared with value for each control experiment.

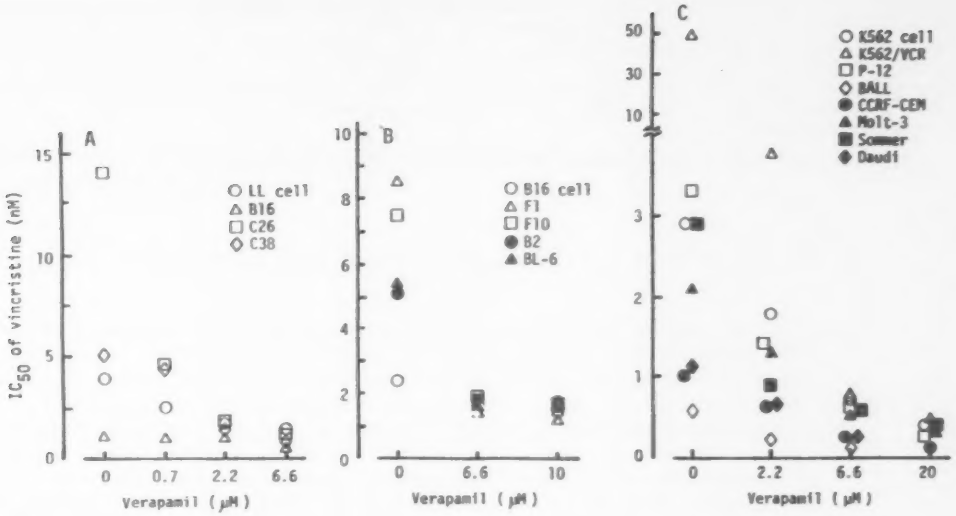

FIGURE 2.—Circumvention of the heterogeneity in VCR sensitivity by verapamil. IC50s of VCR in the absence or presence of verapamil were determined as described previously (6,13) against Lewis lung carcinoma (LL), B16 melanoma (B16), colon adenocarcinoma 26 (C26), and colon adenocarcinoma 38 (C38) in panel A; B16 (parent), F1 variant (F1), F10 variant (F10), B2 variant (B2), and BL-6 variant (BL-6) in panel B; and K562 myelogenous leukemia (K562), its VCR-resistant line (K562/VCR), acute lymphoblastic leukemias of T-cell origin (P-12, CCRF-CEM, Molt-3, and Sommer), acute lymphoblastic leukemia of B-cell origin (BALL), and Burkitt's lymphoma (Daudi) in panel C.

tentiation of drug responsiveness in tumor cell lines inherently resistant to the drug; (*c*) circumvention of the heterogeneity in drug sensitivity; (*d*) effectiveness against various antitumor agents which are transported to the outside of cells by the same mechanisms; and (*e*) dose de-escalation of antitumor agents.

We are currently searching for calcium antagonists and calmodulin inhibitors which may prove to be effective clinically in cancer chemotherapy. At present, we have started a pilot clinical evaluation of the reversal of VCR and ADM resistance in patients by using diltiazem and nicardipine. These two calcium influx blockers are now used clinically as coronary vasodilators, and these two drugs possess rather potent activity for the reversal of acquired resistance to VCR and ADM in animal experiments.

The mechanism of the present approach is not yet known. It seems to be reasonable that the functions of cellular calcium and calmodulin are involved in drug efflux mechanisms in resistant tumor cells. Elucidation of the mechanisms will provide better ways to reverse acquired resistance to vinca alkaloids, anthracycline antibiotics, and other related compounds.

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Enhancement of Doxorubicin and Vinblastine Sensitivity in Anthracycline-Resistant P388 Cells

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Acquired resistance to doxorubicin in a P388 murine leukemia cell subline was found to be associated with decreased drug accumulation in these cells. We have previously shown that the lipid domain of the plasma membrane in drug-resistant cells is structurally more ordered and has a lower phosphatidylcholine/sphingomyelin ratio. Perhexiline maleate and triparanol both markedly enhance the sensitivity of drug-resistant cells to doxorubicin and vinblastine but do not have an effect on anthracycline-sensitive cells. This enhanced sensitivity is associated with increased drug accumulation. Although perhexiline maleate has been reported to be a calcium antagonist in other systems, our data do not implicate this mechanism in the enhancement of cell sensitivity to doxorubicin. We suggest that this effect might be related to alterations of the cell membrane lipid domain induced by perhexiline maleate and triparanol, which result in decreased structural order of plasma membrane lipids and permit increased drug accumulation. [Cancer Treat Rep 67:895–899, 1983]

To investigate the mechanism of acquired resistance to anthracyclines, we have carried out comparative studies using P388 cells and a doxorubicin-resistant P388 subline (P388/ADR) maintained continuously in suspension culture.

As reported by Inaba and Johnson (1), we have observed reduced accumulation of doxorubicin in P388/ADR cells compared to the sensitive parent cell line. However, the doxorubicin uptake data we obtained do not support carrier-mediated drug transport in these cells. Similar results with regard to drug uptake were reported by Dalmark and Strom (2), who suggested that the transport of anthracyclines takes place by simple Fickian diffusion through the lipid domain of the cell membrane. The rate of doxorubicin entry into cells would therefore be expected to depend, in part, on the ease of molecular motion in the lipid phase of the cell membrane. Measurements of cell membrane lipid structural order, using electron spin resonance spectroscopy following incubation of cells from both lines with the *N*-oxyl-4′-4′-dimethyloxazolidine derivative of 5-ketostearic acid, revealed a higher degree of structural order of the membrane lipids of P388/ADR cells compared to doxorubicin-sensitive cells. Steady-state fluorescence depolarization measurements, following incubation of cells from these lines with 1,6-diphenyl-hexatriene, also indicated more restricted motion of the probe in doxorubicin-resistant cells (3).

The structural order of the cell membrane lipid domain may be determined by membrane lipid composition. Analysis of the lipid composition of cells of both lines revealed that the phosphatidylcholine/sphingomyelin ratio was 5.57 in drug-sensitive cells and 3.28 in drug-resistant cells.[4] Upon staining with Oil Red O, we observed that P388/ADR cells contained a significantly larger number of intracellular lipid vacuoles than drug-sensitive cells (3). This intracellular lipid was found to be mainly triglyceride (3).[4] These differences in lipid composition, as well as the slower rate of incorporation of [^{3}H]choline into phosphatidylcholine in P388/ADR cells, suggest that P388/ADR cells have a lower activity of phosphocholine transferase than drug-sensitive P388 cells.[4] Reduced activity of this enzyme in P388/ADR cells may result in the observed changes in lipid composition and the higher structural order of membrane lipid domains in these cells. These changes in the cell membrane result in a

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[3]We thank Dr. Jacqueline Whang-Peng for her advice and instruction in performing the cytogenetic studies of these cells.

[4]Ramu A, Glaubiger D, and Weinstraub H. Differences in lipid composition of adriamycin sensitive and resistant P388 cells. Submitted for publication.

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TABLE 1.—% of marker chromosomes in P388 and P388/ADR leukemia cell lines*

Cell line	% of marker chromosomes					
	M1a	M1b1	M1b2	M2	M6	Minutes
P388	80	0	6.7	0	0	6.7
P388/ADR	100	86.7	6.7	73.3	80	100

* Chromosome 1 markers—M1a, M1b1, M1b2; chromosome 2 marker—M2; chromosome 6 marker—M6.

slower rate of doxorubicin entry and lower sensitivity of these cells to the drug.

When a cell population is subjected to selection pressure, such as exposure to a cytotoxic drug, sublines that are selected may have a number of genetic changes. It is possible that only some of these changes are related to the cell's ability to survive under the selection pressure, while others may result in characteristics unrelated to that mechanism.

Differences in content of certain proteins and glycoproteins between drug-sensitive and drug-resistant cells have been presented by other speakers at this symposium. We have also noted other differences between these cell lines, including differences in activity of catalase (4), 5′-nucleotidase,[5] transglutaminase,[5] and arachidonate metabolism.[5] However, it remains unclear whether any or all of these changes are related to the mechanism of resistance to doxorubicin.

Karyotypic analysis of both these cell lines revealed specific structural and numerical chromosomal differences between them (table 1; figs 1 and 2). P388/ADR cells had unique marker chromosomes M1b1, M2, and M6 that were not found in P388 cells, whereas both cell lines contained marker chromosomes M1a, M1b2, and minute chromosomes. Unlike resistance to methotrexate (5,6), it was not possible to relate the karyotypic changes to a specific enzyme activity, and, in fact, both cell lines show similar sensitivity to methotrexate.

One way to decide whether a certain difference between drug-sensitive and drug-resistant cells is related to the mechanism of resistance would be to treat the cells in some way that would reverse that change, and see whether it was also associated with reversal of resistance to the drug.

To test our hypothesis that the observed changes in cell membrane lipids in P388/ADR cells are related to their lower sensitivity to doxorubicin, we have studied whether compounds known to affect cell lipid metabolism change the sensitivity of cells to doxorubicin. Results obtained in studies with perhexiline maleate, a drug that was recently reported to change the content of phospholipids in fibroblasts (7), are presented in table 2. As shown, at noninhibitory concentrations, perhexiline maleate markedly increased the sensitivity of P388/ADR cells to doxorubicin, but did not have such an effect on anthracycline-sensitive cells. This increased sensitivity was associated with an increase in drug accumulation by these cells (8). The effects of perhexiline maleate are reversible, suggesting that the drug must be present concurrently with doxorubicin to exert its effect.

Reduction in cellular cholesterol also results in decreased structural order of cell membrane lipids. Therefore, we have tested the effects of triparanol, an inhibitor of cholesterol synthesis (9), on the sensitivity of both cell lines to doxorubicin. The results are presented in table 3. As shown, the effects observed were similar to those seen with perhexiline maleate.

Other changes in cell lipid composition may result in similar effects. It was recently reported that the addition of phosphatidylinositol to the culture medium of KB cells enhanced the accumulation and cytotoxicity of vinblastine (10). Anthracycline-resistant cells are known to be cross-resistant to vinca alkaloids. We have demonstrated that perhexiline maleate greatly enhanced the sensitivity of P388/ADR cells to vinblastine and that this effect was also associated with increased drug accumulation (8).

The exact mechanism by which perhexiline maleate affects the lipid metabolism of cells is not known. One possibility is that the change in lipid composition is secondary to a direct effect of the drug on the cell membrane. Such an effect could directly influence doxorubicin uptake, as was shown for the detergent Tween 80 (1). Another possibility is related to calcium antagonistic effects shown for perhexiline maleate in another system (11). Tsuruo et al (12) have reported that verapamil, another calcium antagonist, enhanced the accumulation and cytotoxicity of vincristine in P388 cells. They speculated that verapamil blocked a calcium-dependent vincristine efflux mechanism in a manner similar to the effects of verapamil on hormonal secretion (13). To test whether the effects of perhexiline maleate on the sensitivity to doxorubicin are related to calcium antagonism, we measured the doxorubicin sensitivity of both lines under conditions where the calcium concentration in the medium was lowered to one-tenth of the standard level or raised progressively up to 1×10^{-2} M. No change in the sensitivity to doxorubicin was observed for either line, and there was no change in the effect of perhexiline maleate. No change in the responses of these cells to drugs was observed in

[5] Ramu A, Glaubiger D, Soprey P, et al. Differences in some plasma membrane related activities between adriamycin sensitive and resistant P388 cells. Submitted for publication.

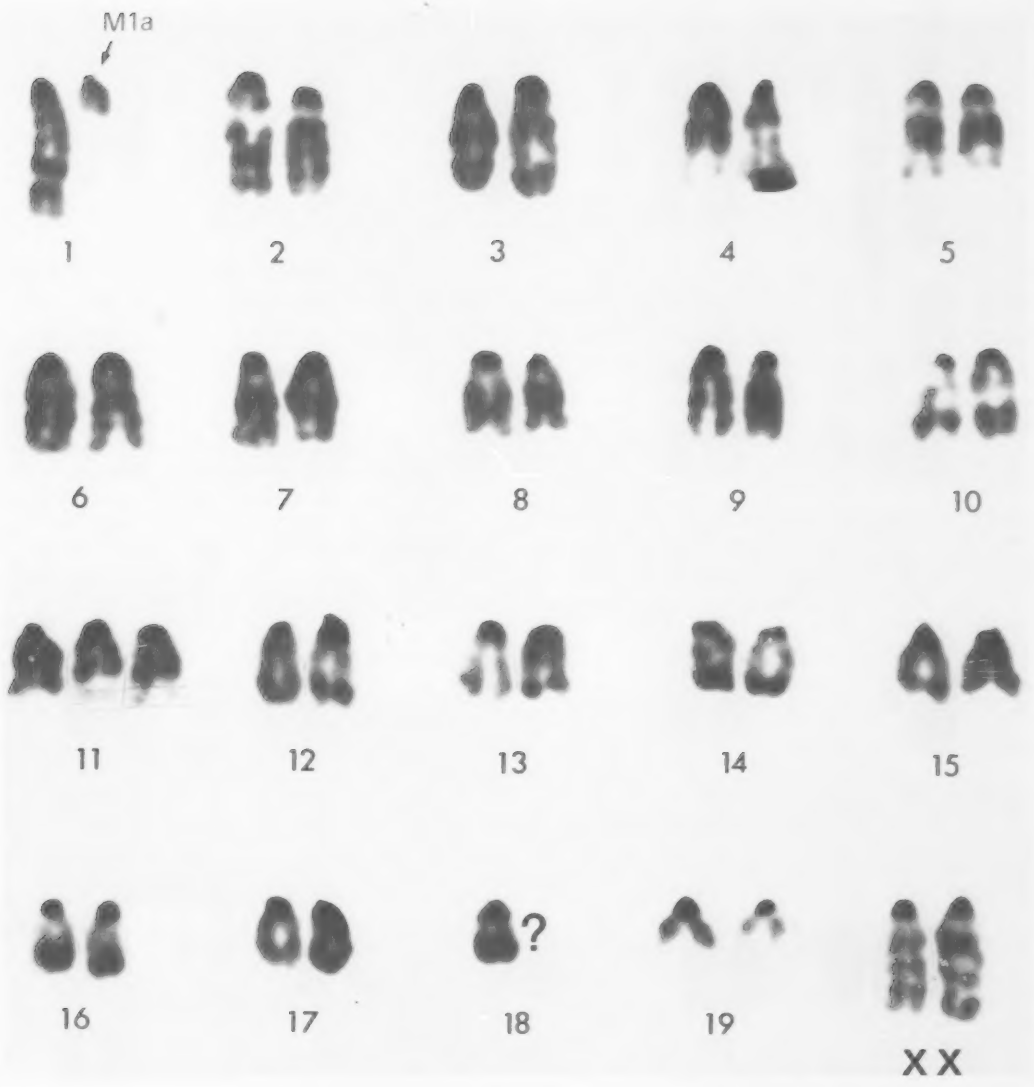

FIGURE 1.—Representative karyotype of P388 murine leukemia cells showing 40 chromosomes, including 1 marker, M1a (Giemsa-trypsin banding).

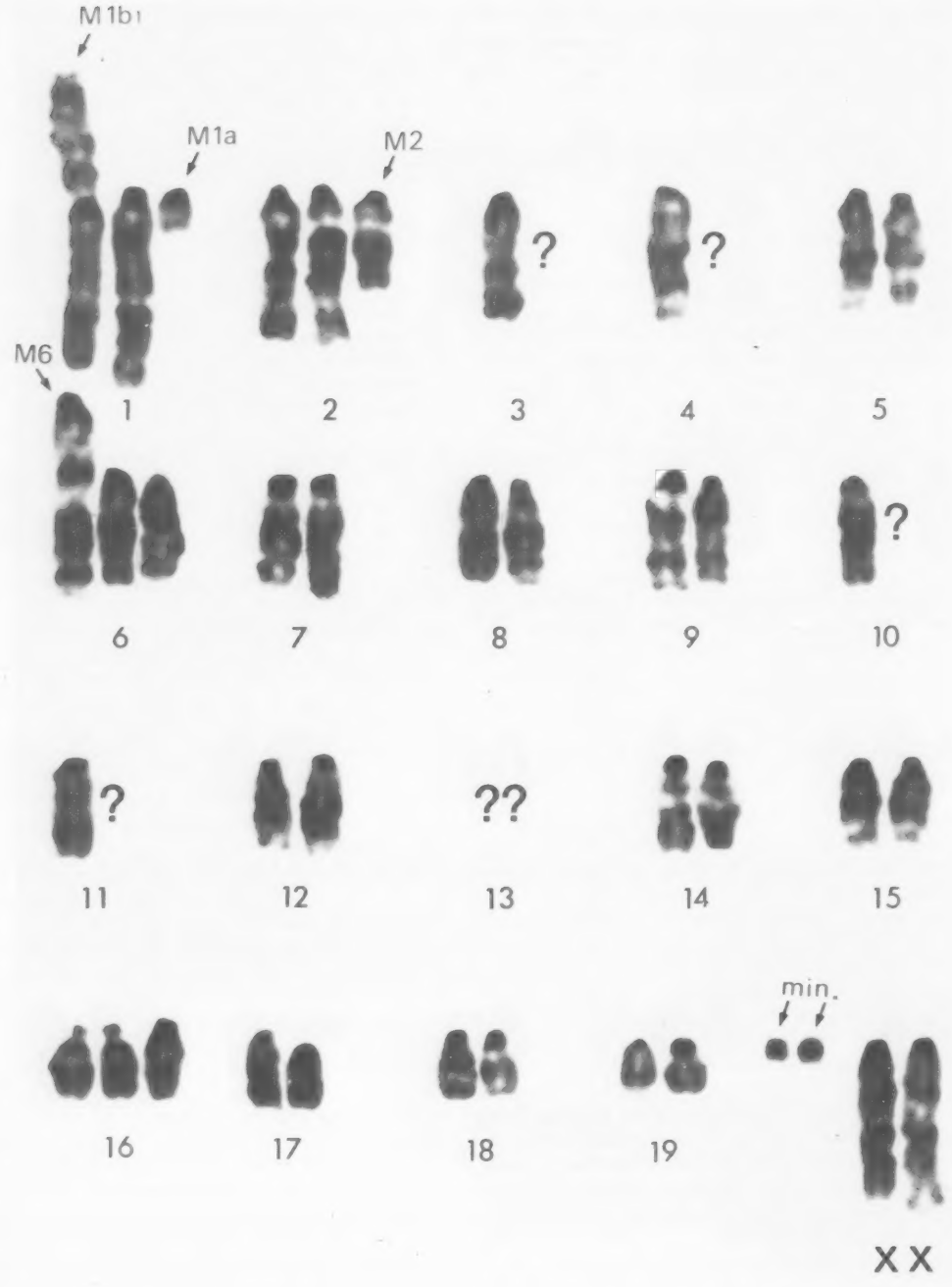

FIGURE 2.—Representative karyotype of P388/ADR cells showing 40 chromosomes, including 5 markers, M1a, M1b1, M2, M6, and minutes (min.) (Giemsa-trypsin banding).

TABLE 2.—Effects of 1×10^{-5} M perhexiline maleate (PM) on the sensitivity of P388 and P388/ADR cells to doxorubicin

Doxorubicin concentration (M)	% of control growth rate			
	P388	P388 + PM	P388/ADR	P388/ADR + PM
—	100	100	100	100
1×10^{-8}	101	101	101	95
3×10^{-8}	69	68	98	90
6×10^{-8}	59	47	98	95
1×10^{-7}	38	46	101	71
2×10^{-7}	20	8	101	15
3×10^{-7}	8	10	98	7
6×10^{-7}	1	2	84	2
1×10^{-6}	0	0	66	0
3×10^{-6}	0	0	9	0

the presence of EGTA or the calcium ionophore A23187. Finally, indomethacin, another drug reported to be a calcium antagonist (14), and lanthanum ions, which compete with calcium ions for the same channels in excitable membranes, did not affect the sensitivity of either cell line to doxorubicin. Thus, our data do not support the suggestion that calcium antagonism is involved in the enhancement of doxorubicin cytotoxicity in drug-resistant cells by perhexiline maleate. Rather, we suggest that drugs that can decrease the structural order of the cell membrane lipid domain, either by a direct effect or through changing the membrane lipid composition, may enhance the entry of drugs like anthracyclines and vinca alkaloids into resistant cells and thus enhance their cytotoxicity.

TABLE 3.—Effects of 3×10^{-6} M triparanol (T) on the sensitivity of P388/ADR cells to doxorubicin

Doxorubicin concentration (M)	% of control growth rate	
	P388/ADR	P388/ADR + T
—	100	98
3×10^{-8}	98	92
6×10^{-8}	103	74
1×10^{-7}	96	46
2×10^{-7}	93	10
4×10^{-7}	83	1

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Gene Amplification and Altered Enzymes as Mechanisms for the Development of Drug Resistance[1]

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Two known mechanisms by which neoplastic cells may become resistant to chemotherapeutic agents are reviewed, using methotrexate (MTX) resistance as a model. These mechanisms are an increased level of target enzyme, found in several instances to be a consequence of gene amplification, or an altered target enzyme or receptor, less capable of binding the drug. An example of MTX resistance due to low-level gene amplification in leukemia cells from an MTX-resistant patient is described. Strategies for selectively eradicating these resistant cell populations may be formulated based on the mechanism by which these cells became drug-resistant. [Cancer Treat Rep 67:901–904, 1983]

Overproduction of the methotrexate (MTX) target enzyme, dihydrofolate reductase (DHFR), has been known to be a cause of MTX resistance in mammalian cells since 1961 (1,2). With the development of modern molecular biology, this increase in enzyme activity has been shown to be due to amplification of the DHFR gene (3). Although less commonly observed, resistance to MTX associated with emergence of an "altered" DHFR, less inhibited by MTX than the parent enzyme, has also been detected in several different MTX-resistant cell lines (4–6). These two mechanisms which result in MTX resistance will be reviewed in this paper, and evidence will be presented that DHFR gene amplification may occur in leukemia cells of patients treated with this drug. The third common mechanism of MTX resistance is impaired MTX transport, which will be discussed elsewhere in this symposium.

Since these mechanisms are not specific for MTX, a review of other drugs that have been found to produce resistance by these mechanisms in mammalian cells will also be presented.

Gene Amplification as a Mechanism of Resistance to MTX and Other Drugs

Several mammalian cell lines resistant to MTX by virtue of DHFR overproduction have been studied in the past several years (3,7–11). In every MTX-resistant subline for which increased levels of this enzyme were found, associated amplification of the DHFR gene has been observed. In general, the degree of gene amplification has correlated with the level of DHFR activity, and cell lines with high numbers of gene copies (200–400) have been obtained that are highly resistant to MTX. These lines have also overproduced DHFR mRNA and have proven to be extremely valuable for the study of gene organization and mRNA formation and regulation.

Also of great interest has been the demonstration that highly resistant, stable, DHFR-overproducing cell lines develop a "homogeneous staining region," ie, a portion of the chromosome that lacks banding characteristics or bands abnormally (7,8,11,12). Unstable resistance may be associated with an increased number (over wild-type cells) of small paired chromosomes, lacking a centromere, called double-minute chromosomes (13).

Several human cell lines highly resistant to MTX have recently been developed by exposure to stepwise increases in MTX concentration (9–11, 14). We have recently described a subline of the human leukemia blast cell line K562, which is markedly resistant to MTX and which has a 240-fold increase in DHFR activity (11). This subline, obtained by exposure to stepwise, gradual increases in MTX concentrations, has corresponding elevated DHFR mRNA(s) and DHFR genes. Of interest is the presence of three chromosomes with homogeneous staining regions, not found in the parent line, which we

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have tentatively identified as chromosome Nos. 5, 6, and 19. In studies with mouse and hamster lines resistant to MTX, the homogeneous staining regions were found to be the site of the amplified DHFR gene and, in addition, were associated with a stable karyotype (7,12).

The availability of cDNA DHFR probes, both mouse (15) and human (9), has allowed a study of the size and organization of the DHFR gene from mouse, hamster, and human sources. The human gene, like the mouse gene, is very large (about 30 Kb) and has at least four intervening sequences (16). By using both the mouse and human cDNA probes, we have noted significant sequence diversity in the 3′ end of the gene, in the noncoding region. The mouse, hamster, and human MTX-resistant lines produce multiple DHFR mRNAs (11,17,18). In the case of the Chinese hamster lung line, two different DHFR enzymes have been noted (17). No evidence exists for more than one species of DHFR produced in the mouse and human resistant lines. In contrast to the mouse DHFR mRNAs, in which the predominant cytoplasmic mRNA is 1.6 Kb in size (17), the major human DHFR mRNA is 3.8 Kb (9,11).

Gene Amplification in Patients Treated With MTX

Since MTX-resistant human cell lines have been described as a result of gene amplification, an important question is whether or not MTX resistance occurs in the clinic by this mechanism. In some instances, leukemia cells from patients whose disease was clinically resistant to MTX could also be documented to be biochemically resistant to MTX by measuring the effect of MTX on [^{3}H]deoxyuridine incorporation into DNA in these leukemia blast cells (19,20).

Although leukemia cells from patients treated with MTX were found to have elevated DHFR (21), our previous finding that MTX causes an increase in this enzyme, presumably by protection from degradation (22), made it difficult to determine if DHFR overproduction was the cause of resistance. At that time, techniques for measuring gene amplification were not available.

Recently, we have had the opportunity to study in detail a patient with acute myelocytic leukemia, whose disease was highly resistant to MTX treatment (23). This patient had several remissions induced by daunorubicin, cytarabine, and 6-thioguanine, and despite treatment during remission with several drugs including MTX, he eventually became refractory to reinduction with these and other drugs, including 5-azacitidine and amsacrine. Resistance to MTX was established by lack of leukemia cell response to a 24-hour infusion of MTX.

Examination of the patient's leukemia blasts showed an increase in DHFR activity (threefold to sixfold higher than normal), with no evidence of altered affinity of the enzyme to MTX. By using a "dot blot" assay that we developed to be accurate enough to detect as small a difference as one gene copy, we found that this patient had a fourfold increase in DHFR gene copy number.

The DNA from the blast cells of this patient was analyzed by restriction analysis. When restriction digests were probed with a human DHFR cDNA probe (9), an increase in the signal for DHFR coding sequences was noted as compared to Southern blots from leukemia cells not resistant to MTX (23). The major bands previously detected in the human leukemia K562 sensitive and resistant to MTX were observed. Additional bands were also noted; these bands may arise from amplification of pseudogenes, restriction site anomalies, or heterogeneity within the malignant cell population.

The enzymatic and genetic analysis gives support to gene amplification as the mechanism of MTX resistance in this patient. Our preliminary examination of the patient's karyotype did not reveal either homogeneous staining regions or double-minute chromosomes.

Altered DHFR as a Mechanism for MTX Resistance

Although resistance to MTX as a consequence of alteration of DHFR occurs less frequently than an elevated DHFR or impaired transport of MTX, several lines have been described with this alteration (4-6,24,25). In bacteria, resistance to trimethoprim has been shown to occur commonly by virtue of alteration of the bacterial DHFR, or R-factor DHFR (26,27). In a resistant subline of *Escherichia coli* resistant to MTX, the alteration in the enzyme was found to be associated with a substitution of an arginine for a leucine in position 28 (26). This change caused a marked decrease in binding of 4-amino analogs to the enzyme, while still permitting enzymic catalysis with the normal substrate. Haber and Schimke recently

TABLE 1.—Examples of drug resistance in mammalian cells associated with increased production of an enzyme or receptor

Drug	Enzyme or receptor	Gene amplification	Ref No.
MTX	DHFR	Yes	3
PALA	CAD enzyme	Yes	35
Hydroxyurea	Ribonucleotide reductase	?	36
5-Fluorodeoxyuridine	Thymidylate synthetase	?	37
Deoxycoformycin	Adenosine deaminase	Yes	38
L-Asparaginase	Asparagine synthetase	Yes	39
Ca^{++}, ZN^{+}	Metallothionein	Yes	40

TABLE 2.—Examples of drug resistance in mammalian cells associated with production of an altered enzyme or receptor

Drug	Enzyme or receptor	Ref No.
MTX	DHFR	20
Hydroxyurea	Ribonucleotide reductase	41
5-Fluorodeoxyuridine	Thymidylate synthetase	42
Demecolcine	β-Tubulin	43
Hydrocortisone	Cytoplasmic receptor	44

described a mouse 3T6 subline with an elevated and altered DHFR; for 50% inhibition, this enzyme required a concentration of MTX several logs higher than that required in the parent line (20). We have developed a selective elution technique using an affinity column of MTX-sepharose to separate the altered and normal DHFR from these cells (28). It will be of great interest to sequence this enzyme to determine the change in the amino acid sequence that is associated with decreased binding of MTX to the enzyme.

Of potential value is the use of altered drug-resistant genes, such as the altered DHFR, to transfer MTX resistance to other cells, including normal bone marrow (29,30). These genes may also be used for cotransformation studies, in which another gene is used together with the DHFR gene for transfection; cells incorporating these genes may be selected by subsequent exposure to MTX (31).

Gene Amplification or an Altered Target Enzyme as Mechanisms of Resistance to Other Drugs

Since the initial observation that MTX resistance in mammalian cells was due to gene amplification, other examples of enzyme or receptor overproduction associated with resistance to other drugs have been reported. In some of these instances, gene amplification has been found to be responsible for these changes (table 1). Thus, it is clear that gene amplification is not a phenomenon associated uniquely with MTX resistance but occurs as a consequence of resistance to other drugs as well. It seems evident that neoplastic tumor cell populations can respond to drug decimation by survival of cells, presumably pre-existant mutants, that have an increased number of gene copies coding for the drug target. The mechanism by which cells amplify these genes is yet not elucidated (32). Of interest is the apparent inability of normal cell populations to develop drug resistance.

DHFR alteration, unless induced by treatment with mutagens, is a less common mechanism of MTX resistance than is amplified DHFR. Resistance to other drugs can also result from other presumed gene mutations leading to altered drug affinities for enzymes or receptors (table 2). These observations, in particular with DHFR, may provide important insights into understanding drug-target binding.

Clinical Implications

The understanding of drug resistance at the molecular level may lead to approaches that prevent or delay these phenomena. Documentation of resistance to a drug will also provide helpful information to the physician, who may then spare the patient from the toxicity of further treatment with a relatively ineffective agent.

A strategy for chemotherapy based on knowledge of the mechanism of drug resistance in cells that survive treatment with a chemotherapeutic agent may be formulated, ie, the use of a drug or of drugs that *selectively* kill resistant cells emerging as the consequence of initial treatment. For example, after initial remission induction of patients with MTX or MTX-containing regimens, if disease recurs during MTX treatment and the mechanism is documented, specific treatments that may be relatively nontoxic may be directed toward these MTX-resistant cells. For example, in the case of increased DHFR activity, the patient may be treated with drugs that are substrates for DHFR, and then become inhibitors of thymidylate synthetase (33); other strategies based on knowledge of resistance may be similarly developed (34).

Studies of resistance to MTX in this and other laboratories have provided evidence that DHFR gene amplification occurs in neoplastic cells of patients treated with MTX (45,46). Of importance will be further studies that establish the frequency of this event as well as other mechanisms of resistance to MTX in patients.

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Establishment of Cross-Resistance Profiles for New Agents[1]

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Sublines of murine leukemias (L1210 and P388) and solid tumors selected for resistance to representatives of all of the chemical and functional classes of clinically useful anticancer drugs have been isolated and established in serial in vivo passage and, in some cases, in vitro culture. Extensive resistance, cross-resistance, and collateral-sensitivity patterns have been established with most of the sublines of the drug-resistant murine leukemias under treatment with > 100 different established and clinically useful anticancer drugs or new candidate anticancer drugs currently under study. Patients selected for inclusion in phase I–II trials usually have tumors that have failed to respond to treatment with established clinically useful drugs, either from the start of treatment or during continuing treatment after initial useful response. These treatment failures are no doubt due, in many cases, to drug-resistant tumors if initially unresponsive or to the overgrowth of drug-resistant mutant tumor stem cells in initially responding patients who ultimately failed under continuing treatment. Therefore, the cross-resistance profiles of drug-resistant murine tumors to treatment with new drugs going into phase I–II trials should provide useful guides for patient selection for those trials. Also, these cross-resistance profiles will provide useful information indicating likely biochemical mechanism of action of new drugs with promising anticancer activity, thus guiding drug selection for combination chemotherapy trials in animals or man. Numerous examples of all of the above indications for useful application of such information derived from chemotherapy trials with drug-resistant murine tumors are reported. [Cancer Treat Rep 67:905–922, 1983]

It is commonly observed with drug treatment of both leukemias and solid tumors of man and animals that initially drug-sensitive and responsive tumors become progressively less responsive and ultimately fail to respond during continuing treatment (1,2).

Spontaneous mutation to drug resistance is commonly observed among advanced-stage and grossly evident drug-sensitive murine tumors that are used as experimental models for chemotherapy trials and that were selected to represent the major histologic and organ types of human tumors (2). The rate of spontaneous mutation of murine tumors to resistance to single anticancer drugs varies markedly, being highest to mitotic inhibitors like vincristine (VCR) (1,2), less frequent to antimetabolite drugs like cytarabine (ara-C) (1–3), and lowest to highly active drugs like the alkylating agents, eg, cyclophosphamide (CPA) (1,2). Spontaneous mutation to resistance to all chemical and functional classes of anticancer drugs, including the alkylating agents (4,5), has been observed in mice with total-body burdens of tumor stem cells that are at or below the smallest body burden of all organ or histologic types of cancer in man at a time when the tumor is first clinically detectable (about 10^9 tumor cells in a single focus). Even patients without clinically evident tumor at the start of drug treatment, eg, those receiving drug treatment shortly after surgical removal or radiation kill of evident and accessible primary and/or metastatic lesions, would have had a total-body burden of tumor stem cells prior to surgery and/or radiation large enough to establish a high probability of the presence of residual tumor stem cells which could be resistant to any drug and therefore could be a potential obstacle to curative chemotherapy.

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Since drug-resistant tumor stem cells are an indicated obstacle to curative drug treatment of clinically recognized systemic cancer in man and an objectively established obstacle to curative drug treatment of advanced and grossly evident cancer in experimental animals, it is important to develop methods for control of drug-resistant tumor cells.

We and other investigators are studying the drug-resistant problem in experimental cancer chemotherapy.[3]

When a new anticancer drug is selected for clinical trial on the basis of observed anticancer activity against one or more transplantable animal tumors or human tumor xenografts growing in animals, the patient selection for phase I–II testing is often from previously treated patients who did not respond to treatment or who ultimately relapsed under initially effective and useful drug treatment. If the treatment failure was due to overgrowth of tumor cells resistant to the drugs used in initial treatment, new drugs without activity against tumor cells resistant to the inactive or failing treatment could be prospectively predicted to fail also. Therefore, the sensitivity to the new agent of animal tumor cells selected for resistance to clinically useful drugs that initially or ultimately failed to control each individual patient's tumor should be a prime determinant in patient selection for phase I–II trials. If experimental tumors selected for resistance to the drugs to which the patient's tumor failed to respond were cross-resistant to the new drug entering phase I–II trials, that patient probably would fail under treatment with the new drug in clinical trials, and if a sufficient number of such patients were included in the trials, an otherwise promisingly useful anticancer drug could be overlooked and abandoned.

Studies with animal tumor cells with known drug resistance have provided in the past, and can provide in the future, useful information on all of the basic phenomena relating to drug resistance, biochemical mechanism of action of new drugs, etc. Included in the knowledge to be gained from such studies is an understanding of the mechanism(s) by which originally drug-sensitive tumor stem cells become resistant to drug treatment: eg, enzyme deletion, membrane transport and intracellular retention of drugs, gene amplification resulting in increased formation of the target enzymes, comparison of cytotoxic moieties of alkylating agents, increased DNA repair in drug-resistant tumor stem cells, and increased levels of degradative enzymes, all of which may bear directly and individually or collectively on anticancer activity of the new drug in animals and/or man.

[3]Different techniques and procedures are used by different investigators to collect and interpret data from drug-resistance studies. Since we are only responsible for our own data, we are knowingly, and with apologies to other investigators, limiting this report, with few exceptions, to data that have been collected at Southern Research Institute.

Drug-Resistant Murine Leukemias and Solid Tumors Isolated at Southern Research Institute and Available for Study

Drug-resistant sublines of leukemias L1210 and P388 are shown in table 1, and sublines of murine solid tumors that we have isolated under treatment with representatives of all of the currently recognized chemical and functional classes of clinically useful anticancer drugs are shown in table 2. Log_{10} changes in the body burden of the parent drug-sensitive (L1210/0 and P388/0) and the drug-resistant sublines of L1210 and P388 are shown in table 3. In tables 4 and 5, we have tabulated the results of extensive, but still far from complete, chemotherapy trials in which we treated the drug-resistant tumor sublines in the same experiment, in direct "head-to-head" comparison with the parent drug-sensitive tumor, using multiple doses of each drug under study, so that optimal therapeutic response of

TABLE 1.—Murine leukemias selected for resistance to clinically useful anticancer drugs

	Resistant to—*	
	L1210	P388
Alkylating agents		
CPA	X	X
Melphalan	X	X
Carmustine	X	X
Cisplatin	X	X
Antimetabolites		
Ara-C†	X	X
Hydroxyurea	X	
Thiosemicarbazone‡	X	
5-FU		X
5-Azacitidine (Azacyt)		X
6-Thioguanine (6-TG)	X	
6-Mercaptopurine (6-MP)	X	
6-Methylmercaptopurine riboside (6-MeMPR)	X	
Tiazofurin§		X
Ara-A		X
Methotrexate (MTX)		X
DNA binders or intercalators		
Doxorubicin (ADR)		X
Dactinomycin (Act D)		X
Amsacrine (*m*-AMSA)¶		X
Mitotic inhibitor		
VCR		X
Doubly resistant		
CPA and ara-C	X	
Ara-C and 6-TG	X	
Ara-C and 6-MP	X	
Ara-C and 6-MeMPR	X	
6-MP and 6-MeMPR	X	

*X indicates that drug-resistant tumors have been adapted to growth in cell culture and are currently available.

†We also have an ara-C-resistant mutant of murine acute myelogenous leukemia (AML) (RFM).

‡Pyridine-2-carboxaldehyde thiosemicarbazone.

§2-β-D-Ribofuranosylthiazole-4-carboxamide.

¶Isolated by R. K. Johnson.

TABLE 2.—Drug-resistant sublines of murine solid tumors isolated at Southern Research Institute during continuing treatment with drugs that initially caused regression of grossly evident (advanced) tumors

Drug	Tumor
Alkylating agents	
L-PAM	M5076 ovarian
Semustine (MeCCNU)	M5076 ovarian
Dacarbazine (DTIC)	Colon 07
DDPt	Colon 04/C
DNA binder or intercalator	
ADR	Mammary 16/C
ADR	Mammary 17/A
Antimetabolites	
Ara-C	Colon 36
5-FU	Colon 12
Triazine antifol (NSC-127755)	Colon 36

≤ LD10 doses, based on change in the body burden of the stem cells of both the drug-resistant and the parent drug-sensitive tumor lines, could be made. The technical procedures and the methods of estimating changes in the body burden of tumor stem cells have been described (7) and previously reported (2,8). Simply stated, the number of tumor stem cells present at the start and at the end of drug treatment are estimated from a plot of the mortality and median lifespan of untreated control mice implanted with $\log_{10}$ dilutions of tumor cells from 10^7 down to one cell, with both the parent drug-sensitive and the indicated drug-resistant sublines in each experiment. From such plots, one can estimate the number of tumor stem cells present at the start of and at the end of drug treatment, irrespective of size of the tumor implant, the duration of treatment, or other characteristics of the treatment schedule used. In our opinion, this is the most objective and quantitatively precise and reproducible method of estimating therapeutic effectiveness of drug treatment: ie, the change in the body burden of tumor stem cells observed under drug treatment at up to dose-limiting toxicity. Increase in lifespan (ILS), as it is commonly used as an endpoint for estimating therapeutic activity of drug treatment, commonly disregards the duration of treatment and does not provide objective estimates of the body burden of tumor stem cells at the end of drug treatment. If reduction of the body burden of tumor stem cells to below the number capable of re-establishing the ultimately fatal disease is the goal of cancer chemotherapy, as we believe it is, then such estimates of the change in body burden of tumor stem cells by drug treatment (in the absence of cure) are essential for objective evaluation of the therapeutic activity of any drug, drug combination, or treatment protocol.

TABLE 3.—Log_{10} change* in body burden of tumor stem cells after optimal (≤ LD10) drug treatment

	L1210/0	L1210/drug-resistant	P388/0	P388/drug-resistant
Alkylating agents				
CPA	-6	0	-7	-1
L-PAM	-6	-2	-7	-1
BCNU	-7	-2	-7	-1
DDPt	-5	-1	-6	-2
DNA binders or intercalators				
ADR	-3		-6	-2
Act D	-1		-5	-2
m-AMSA	+1		-6	+3†
Mitotic inhibitor				
VCR	+4		-6	-2
Antimetabolites				
Ara-C	-6	+1	-6	-1
Hydroxyurea	-4	+3		
Thiosemicarbazone	-5	+3		
5-FU	+1		-5	+2
Azacyt	-5		-6	+3
6-TG	-3	0	-3	
6-MP	-2	+1	0	
6-MeMPR	-4	-1	-1	
Tiazofurin	-3		-3	+2
Ara-A	-4		-6	+2
MTX	0		-3	+3

*Log_{10} change = net $\log_{10}$ change in tumor stem cell population at the end of treatment as compared to the start of treatment; eg, a -3 log change means that there was a 99.9% reduction and a +3 log change means that there was a 1000-fold increase in tumor burden at the end of treatment.

†Data of Johnson and Howard (ref 6).

TABLE 4.—Log_{10} change[a] in the body burden of parent drug-sensitive and selected drug-resistant leukemia L1210 stem cell populations by drug treatment (at ≤ LD10 dose from dose-response studies) of BDF_1 or CDF_1 mice implanted ip with 10^5–10^6 tumor stem cells and treated ip as indicated

Agent	NSC No.	Treatment schedule[b]	L1210						
			Parent/0	/CPA	/BCNU	/L-PAM	/DDPt	/Ara-C	/6-TG	/6-MP
Alkylating agents										
Mechlorethamine	762	A	-3			-3				
CPA	26271	A	-6	0	-6	-4	-6	-6		
Ifosfamide (IFA)	109724	A	-7	-2						
4-Hydroperoxyisophosphamide	227114	A	-7	-2‡						
4-Hydroxyisophosphamide	208841	A	§	-2‡						
4-Peroxycyclophosphamide	176986	A	-7	-1‡						
4-Hydroxycyclophosphamide	196562	A	-4	0‡						
Phosphoramide mustard (PM)	69945	A	-6	-5		-4				
Isophosphoramide mustard (IPM)	297900	A	-6	-6‡						
L-PAM	8806	A	-6	-5	-6	-2	-4			
Peptichemio	247516	A	-4‡	-3‡	-2‡	0‡				
Asaley	167780	A	-2			0				
Chlorambucil	3088	D	+2‡	+2‡						
Uracil mustard	34462	A	-3	-2						
Piperazinedione	135758	A	-5	-4	-4	-5	-5			
		D	-2	+2‡	0	-5		0‡		
Thiotepa	6396	A	-5	-2	-2	-2	-3			
Mitomycin	26980	A	-4	-5‡		-3				
Dianhydrogalactitol	132313	A	-5	-5	-5	-4	-4			
		B	-5			-2				
BCNU	409962	A	-7	-7	-2	-6	-7	-6		
Lomustine	79037	A	-7	-6	-2	-7	-7			
MeCCNU	95441	A	-7	-7	-3	-7	-7			
PCNU	95466	A	-7			-7				
Streptozocin	85998	A	0	-1	-1	0				
Chlorozotocin	178248	A	-7	-6	-4	-6	-6			
BIC	82196	A	-7	-6	0	-6	-6			
DTIC	45388	A	-2‡		-1‡					
		D	+1			+2				
Platinum										
DDPt	119875	A	-5	-3‡	-5		-1			
		B	-3				+3			
		C	-5	-5		0	+3			
		D	+2				+3			
155	194814	A	-5‡				-5‡			
(Ethylenediamine)-(malonato)-	146068	D	+2‡				+3‡			
Cyclobutane	241240	D	+2‡				+3‡			
Di-isopropylamine	256927	C	-1‡	-2‡	+1‡	+2‡				
		D	+1‡				+2‡			
Dicyclopentylamine	170898	C	-3‡	+1‡	+2‡	+3‡				
Carboxyphthalato-1,2-diaminocyclohexane	271674	A	-6			-4	-4‡			
		B	-6				-7			
		D	-6			-7	-7			
Dichloro(2, 2-dimethyl-1,3-propanediamine-*N*, *N'*), (SP-4-2)-	349946	B	+2‡				+3‡			

Indicine-*N*-oxide	132319	A	0	+2‡					
Procarbazine	77213	D	+1	+2	+2	+3			
DNA binders or intercalators									
ADR	123127	A	-3			-3			
Act D	3053	A	-1			-2			
Antimetabolites									
MTX	740	C	-2						-6
		D	0	0‡	+2‡	+3	+1‡	-3	-4
Dichloromethotrexate	29630	D	-1						-5‡
10-Deaza-aminopterin	311469	D	+2‡						-4‡
5-Methyltetrahydrohomofolic acid (5-MeTHHF)	139490	D	+3						+2
Quinazoline antifolate	327182	C	+1						+1
2,4-Quinazolinediamine, 5-methyl-6-[[(3,4,5-trimethoxyphenyl) amino]methyl]-, 2-hydroxy-ethanesulfonate (1:1)	328564	D	+2‡						-2‡
L-Glutamic acid, *N*-[4-[[(2,4-diamino-6-pyrido[2,3-*d*]pyri-midinyl)methyl]methylamino] benzoyl]-, dihydrate	344280	C	-1‡						-4‡
L-Glutamic acid, *N*-[4-[[(2,4-diamino-6-pyrido[2,3-*d*] pyrimidinyl)methyl]amino benzoyl]-, diethyl ester	346890	C	-1‡						-6‡
Baker's antifol	139105	D	+4						+3‡
Triazine antifol	127755	D	+3						+3
DDMP	19494	D	+3						+3
6-MP	755	A	-2				-2	-1‡	+1
		D	+3	+3	+3			+3	+4
6-MeMPR	40774	A	-4					-3‡	-1‡
		D	+1					+2‡	+2‡
6-TG	752	A	-3				-3	0	
		D	+3	+3		+4			
Ara-A + 2′-deoxycoformycin (2′dCF)	404241 218321	E	-4				-1		-5
2-Fluoro-ara-A	118218	D	-5				+4		
2-Fluoro-ara-AMP (sodium)	328002	A	-3‡				-3‡		
		D	0‡				+2		
5′-Formyl-2-F-ara-A	302067	D	-2				+4		
Ara-C	63878	E	-6		-6		+4		
PalmO-ara-C	135962	A	-6	-6	-5	-6	+1		
		D	-6						-6
5-FU	19893	D	+1	+1‡	+2‡	+1	+2		+2
Floxuridine (5-FUdR)	27640	D	+3	+3‡	+3‡				
6-Azauridine	32074	D	+3				+4		
3-Deazauridine	126849	D	+2				-6		
		E	+2				-6		
5′-*O*-Acetyl-3-deazauridine	329943	D	+2				-6		
5′-*O*-Propionyl-3-deazauridine	329944	D	+1				-6		
5′-*O*-*N*-Butyryl-3-deazauridine	329945	D	+2				-6		
5′-*O*-Pivalyl-3-deazauridine	329946	D	+2				-6		

TABLE 4.—*Continued*

Agent	NSC No.	Treatment schedule†	L1210							
			Parent/0	/CPA	/BCNU	/L-PAM	/DDpt	/Ara-C	/6-TG	/6-MP
5′-*O*-Benzoyl-3-deazauridine	329947	D	+2					-6		
2′,3′,4′-Tri-*O*-acetyl-3-deazauridine	329948	D	+2					-6		
4′-*O*-(Adamantan-1-carbonyl)-3-deazauridine	329949	D	+2					-6		
5′-*O*-Palmitoyl-3-deazauridine	340570	D	+2					-6		
5-Azacitidine	102816	D	-5			-5		-5‡		
Dihydro-5-azacitidine	264880	D	-2‡					-5‡		
PALA	224131	D	+4					+4		+3
Pyrazofurin	143095	D	+3‡					+3‡		
Hydroxyurea	32065	E	-4§					-4§		
Guanazole	1895	E	-4§					-4§		
P-2-TSC	729	E	-5					-1		
Mitotic inhibitor										
VCR	67574	B	+4			+4				

*Log_{10} change = net log_{10} change in tumor stem cell population at the end of treatment as compared to the start of treatment; eg, a -3 log change means that there was a 99.9% reduction and a +3 log change means there was a 1000-fold increase in tumor burden at the end of treatment.

†A = single-dose; B = q4d × 3; C = qd 1-5 days; D = qd 1-9 days; and E = q3h × 8 and q4d × 3.

‡Single experiment.

§10^4 tumor stem cell implant.

Resistance, Cross-Resistance, and Collateral Sensitivity of Selected Drug-Resistant L1210 and P388 Leukemias to Clinically Useful Anticancer Drugs and to New Drugs Under Development

The data shown in tables 4 and 5 were usually obtained by treatment with the optimal dose (from a dose-response study) and the optimal treatment schedules for the parent drug-sensitive tumor in each case. The compounds listed in tables 4 and 5 are representatives of the clinically useful drugs in each of the chemical and functional classes of anticancer drugs, as well as a number of other compounds that are now or have been under investigation as potentially useful new drugs.

Generally consistent observations among the drug-resistant lines of L1210 and P388 treated with clinically useful anticancer drugs or drugs in development as shown in tables 4 and 5 are as follows.

1. Except for cross-resistance to other drugs with similar chemical structure and/or biologic function, mutation of tumor cells to resistance to one drug usually does not result in resistance to other drugs, particularly those of other functional classes. For example, tumor cells selected for resistance to antimetabolite drugs usually retain full sensitivity to alkylating agents, to drugs that bind to or intercalate with DNA, and to mitotic inhibitors such as VCR.

2. Tumor cells resistant to an anticancer drug are usually cross-resistant to structural congeners of that drug; eg, 6-TG versus L1210/6-MP and congeners of CPA versus L1210/CPA. However, exceptions to this general principle are common. (*a*) L1210/CPA is cross-resistant to a number of analogs of CPA but retains full sensitivity in vivo to PM and IPM. The lack of cross-resistance of L1210/CPA to PM and IPM may be due to increased production of aldehyde dehydrogenase by L1210/CPA (9,10), possibly due to gene amplification. Theoretically, adequate levels of PM and IPM should be formed by normal metabolism of CPA or IFA and, therefore, resistance to CPA would not be evident. Failure of CPA or IFA (at doses up to dose-limiting toxicity) to control CPA-resistant tumor cells is not clearly understood but may be due to different pharmacokinetic circumstances (rate of production of PM from CPA or IPM from IFA), rate of cellular uptake and/or inactivation, or other unrecognized variables. These possibilities are supported by the fact that CPA is markedly cytotoxic in vivo for L1210/CPA at single doses of about 5 times the LD10.[4] (*b*) L1210/DDPt is cross-resistant to most analogs of DDPt but remains very sensitive to the carboxyphthalato analog (NSC-271674) (table 4). The significance of these observations is not clear since both P388/0 and P388/DDPt are essentially insensitive to NSC-271674 (table 5). (*c*) L1210/ara-C and P388/ara-C are sensitive to ara-A + 2′dCF, a potent deaminase inhibitor, but are cross-resistant to 2-fluoro-ara-A on an every day, Day 1-9 treatment schedule, while 2-fluoro-ara-A is active against P388/ara-A (table 5). These activities are to be expected since it is known that 2-fluoro-ara-A is phosphorylated by CdR kinase and not by AdR kinase. Therefore, these failures to predict cross-resistance based on similar chemical structure between ara-A and 2-fluoro-ara-A are well-understood on a biochemical basis (3,8,11).

Collateral Sensitivity

Numerous examples of collateral sensitivity (CS)[5] of L1210 and P388, selected for resistance to antimetabolite drugs, are evident in the data presented in tables 4 and 5. These examples are listed in table 6 for purposes of discussion. The first marked CS reported was that of 6-MP-resistant L1210 cells (L1210/6-MP) to treatment with MTX (13). A similar CS of human leukemia cells may contribute to the clinical effectiveness of VAMP (VCR, MTX, 6-MP, and prednisone) used in treating acute lymphatic leukemia (ALL) of children, although such CS of 6-MP-resistant ALL cells to MTX in man has never been objectively investigated and established. However, CS may be an asset worth investigating and attempting to exploit in relation to the control of ara-C-resistant tumor cells in man. With both L1210/ara-C and P388/ara-C, a number of remarkable examples of CS to other antimetabolite drugs have been observed (table 6).

The quantitatively greatest CS shown in table 6 is that of L1210/ara-C to 3-deazauridine (about 8 orders of magnitude greater cell kill of L1210/ara-C than of L1210/0 at equitoxic doses; ie, at ⩽ LD10 doses). 3-Deazauridine has been tried, without therapeutic response, in patients with AML who had had extensive prior treatment with ara-C and were in relapse, possibly due to overgrowth of ara-C-resistant AML cells. We have tried ara-C plus 3-deazauridine against body burdens of L1210/0 cells large enough to predict treatment failure due to the overgrowth of L1210/ara-C. No therapeutic gain over that from ara-C alone was seen. These clinical and laboratory failures to demonstrate therapeutic improvement associated with CS of ara-C-resistant cells may be due to the greater-than-additive toxicity for vital normal cells when 3-deazauridine and ara-C are used together (*see* ref 3 for a discussion of these clinical and laboratory studies).

L1210/6-MP shows CS to MTX and to some, but not all, new compounds that have been synthesized in drug development programs seeking new and improved

[4]Unpublished data from Southern Research Institute.

[5]Collateral sensitivity has been defined as increased sensitivity of a drug-resistant line of tumor cells to another drug over that seen in the parent drug-sensitive cells (12).

TABLE 5.—Log_{10} change* in the body burden of parent drug-sensitive and selected drug-resistant leukemia P388 stem cell populations by drug treatment (at ≤ LD10 dose from dose-response studies) of BDF1 or CDF1 mice implanted ip with 10^6–10^7 tumor stem cells and treated ip as indicated

Agent	NSC No.	Treatment schedule†	P388												
			Parent/0	/VCR	/ADR	/Act D	/CPA	/L-PAM	/BCNU	/DDPt	/Ara-C	/Ara-A	/5-FU	/MTX	/Azacyt
Alkylating agents															
CPA	26271	A	-7	-7	-7	-7	-1	-7	-6		-6				
IFA	109724	A	-7‡				-4‡								
4-Hydroperoxyisophosphamide	227114	A	-7‡				-6‡								
PM	69945	A	-7				-7	-6							
IPM	297900	A	-6				-6								
L-PAM	8806	A	-7	-7	-7	-7	-7	-1	-6‡	-7‡					
Peptichemio	247516	A	-6				-6‡	-2							
Chlorambucil	3088	D	+1‡				+1‡								
Mitolactol	104800	D	+1‡		+1‡										
Piperazinedione	135758	A	-6	-6	-6	-5	-6	-3	-6‡						
Mitomycin	26980	A	-6				-4	-2	-6						
Dianhydrogalactitol	132313	A	-6				-6	-3	-6						
		B	-6					-1							
BCNU	409962	A	-7	-7	-7	-7	-7	-7	-1						
PCNU	95466	A	-7					-7‡							
ACNU	245382	A	-7					-7‡							
BIC	82196	A	-6				-6	-6‡							
Platinum															
DDPt	119875	A	-6					-2		-2					
		B	-6					+2	-6	+3					
		C	-6	-4	-6	-6	-6			+3					
Pt-155	194814	B	-1‡							0‡					
Carboxyphthalato-1,2-diaminocyclohexane	271674	B	+1‡							+2‡					
Indicine-*N*-oxide	132319	D	+3				0								
Procarbazine	77213	D	+1	+3	+3	+1	+3								
DNA binders or intercalators															
Act D	3053	A	-5	-5	-1	-2	-6	-2							
Azetomycin I	244392	B	-6‡			+1‡									
Azetomycin II	244393	B	-6‡			+1‡									
ADR	123127	A	-6	-5	-2	-5	-6	-5			-6			-6‡	
Daunorubicin	82151	A	-6	0	-1‡										
Zorubicin	164011	A	-6‡		0‡										
AD-32	246131	C	-6	-6											
Aclarubicin	208734	B	0‡		+3‡										
Carminomycin	180024	B	+2‡		+3‡										
Marcellomycin	265211	B	+2‡		+3‡										
Nogamycin	265450	B	+2‡		+3‡		-7				-7				
Anthracenedione	196473	D	-6		+2										
Anthracenedione, diacetate	287513	D	-6		+2		-6								
Anthracenedione, dihydroxyl	279836	A	-7	-6	-1	-6									
		D	-1		+1										
m-AMSA	249992	B	-6	+2‡	+2	-2‡									

Antimetabolites																
MTX	740	C	-3		-2	-3				+1						
		D	-3	-4					-2‡	+3						
Quinazoline antifol	327182	C	0							+1						
2,4-Quinazolinediamine, 5-methyl-6-[[(3,4,5-trimethoxyphenyl)amino]methyl]-, 2-hydroxyethanesulfonate (1:1)	328564	D	-4‡							0‡						
Baker's antifol	139105	D	+2						+2‡							
Triazine antifol	127755	D	-2							0						
10-Deaza-aminopterin	311469	D	-2‡					+2‡	+2							
DDMP	19494	D	+2‡	+2‡												
6-MP	755	A	0													
		D	+2	+2	+2	+2										
6-MeMPR	40774	A	-1													
6-TG	752	A	-3													
		D	+2	+2‡	+2‡	+2‡						-3				
Ara-A + 2'dCF	404241+ 218321	D	-6											+2‡		
		E	-6									0				
L-Alanosine, sodium salt	153353	D	+2									-3		-2		
Tiazofurin	286193	D	-3								-6			+2		
2-Fluoro-ara-AMP (sodium)	328002	A	-3									+2				
		D	-4									+2				
2-Fluoro-ara-AMP (ammonium)	311463	D	-2								-1					
Ara-C	63878	D	-1								-6					
PalmO-ara-C	135962	A	-3	-6	-6	-6					-2‡	-3‡				-3‡
Ara-CDP-diglyceride	—	C	-6								-3‡	-4‡				
5-FU	19893	A	-3		-3							+1‡				
		D	-5	-3	-6	-3					+3					
5-FUdR	27640	A	-3‡				-6	-6			-1	-6	-7	-6‡		
		D	0	-3	-1‡	-2					+2					
3-Deazauridine	126849	D	+3				-1				-6	-5				
5-Azacitidine	102816	A	-3				-1				-6	+1‡	+2			-1
		D	-6								-3‡					+3
Dihydro-5-azacitidine	264880	D	-1													+3
Ara-5-azacytosine	281272	D	-5‡								-1		+3			0‡
PALA	224131	D	+2	+2‡	+2‡							-3‡				
Pyrazofurin	143095	D	+3								-6					
Acivicin	163501	D	-1								-6					
Protein synthesis inhibitors							+2				-3		0			
Homoharringtonine	141633	D	-2	+1	+2						-2		+2			
Bruceantin	165563	D	-2		+1‡						-4					
Mitotic inhibitors											-6					
VCR	67574	A	-6	-2			-2‡									
		B	-6	+2	+3	+3										
Vinblastine	49842	B	-3	0			-6	0		-4	-4‡			-4		
Vindesine	245467	B	-5	+2												
Bis (*N*-ethylidene vindesine) disulfide, disulfate	277096	B	-4	-1						-6‡						
Etoposide	141540	B	-7	-6	+1	-5‡										
Maytansine	153858	E	-6‡	-2‡			-7	-7								

TABLE 5.—*continued*

Agent	NSC No.	Treatment schedule†	P388												
			Parent/0	/VCR	/ADR	/Act D	/CPA	/L-PAM	/BCNU	/DDpt	/Ara-C	/Ara-A	/5-FU	/MTX	/Azacyt
Carbamic acid, 5-amino-1,2-dihydro-3-[[methyl (phenyl)-amino]methyl]pyrido[3,4-*b*]-pyrazine-7-yl, ethyl ester	181928	A	-5	-5‡										-3‡	
Carbamic acid, 5-amino-1,2-dihydro-3-phenyl-pyrido-[3,4-*b*]pyrazine-7-yl-, ethyl ester	330770	A	-4	-3											
Carbamic acid, 5-amino-1,2-dihydro-3-[[(4-methoxy-phenyl) methylamino]methyl]-pyrido[3,4-*b*]pyrazine-, ethyl ester	269416	A	-5‡											-6‡	

* Log_{10} change = net log_{10} change in tumor stem cell population at the end of treatment as compared to the start of treatment; eg, a -3 log change means that there was a 99.9% reduction and a +3 log change means that there was a 1000-fold increase in tumor burden at the end of treatment.

†Schedule: A = single-dose; B = q4d × 3; C = qd 1-5 days; D = qd 1-9 days; and E = q3h × 8 and q4d × 3.

‡Single experiment.

Table 6.—

Sensitivity, Cross-Resistance, and Collateral Sensitivity of Antimetabolite-Resistant Leukemia L1210 and P388

Agent	NSC No.	Log_{10} Change[a] Change in Tumor Stem Cells at End of Optimal Therapy					
		10^5 Stem Cell Implant			10^6 Stem Cell Implant		
		L1210/0	L1210/ARA-C	L1210/6-MP	P388/0	P388/ARA-C	P388/ARA-A
Ara-C (Palmitate)	135962	-6	+1	-6	-6	-1	-6
Hydroxyurea	32065	-4*	-4*				
Guanazole	1895	-4*	-4*				
P-2-TSC	729	-5	-1				
Ara-A + 2'dCF	404241 + 218321	-4**	-1**	-5	-6	-6	+2
2-F-Ara-A	118218	-5**	+4**				
2-F-Ara-AMP	328002				-4**	+3**	-4**
Tiazofurin	286193	-1**		-1**	-3	-6	***
L-Alanosine	153353				+2	-1	
3-Deazauridine	126849	+2	-6		+3	-1	
Dihydro-5-azacytidine	264880	-2**	-5**		-1	-6	
5-FU	19893	+1	+2		-3	-6	
PALA	224131	+4	+4		+2	-3	
Pyrazofurin	143095				+3	-2	
Acivicin	163501				-1	-4	
Homoharringtonine	141633				-2	-6	
MTX	740	0		-4			
Dichloro-MTX	29630	-1		-5**			
3-Deaza-MTX	344280	-1**		-4**			
5-Deaza-aminopterin, Diethyl Ester	346890	-1**		-6**			
10-Deaza-aminopterin	311469	+2**		-4**			
Trimetrexate	328564	+2**		-2**			
5-Methyltetrahydrohomofolic Acid	139490	+3		+2			
Quinazoline Antifol	327182	+1		+1			
Baker's Antifol	139105	+4		+3**			
Triazine Antifol	127755	+3		+3			
DDMP	19494	+3		+3			
CPA	26271	-6	-6		-6	-6	

*10^4 Cell implant.

**Single experiment.

***Examples of collateral sensitivity are shown in the enclosed boxes.

[a]Log_{10} change = Net log_{10} change in tumor stem cell population at the end of Rx as compared to the start of Rx; e g , a -3 log change means that there was a 99.9% reduction and a +3 log change means that there was a 1000-fold increase in tumor burden at the end of Rx.

MTX-like drugs. Data in table 6 indicate that CS of L1210/6-MP separates these new drugs into two obviously different groups, based on both activity against L1210/0 and CS of L1210/6-MP. Perhaps this suggests that new drugs which bind tightly to dihydrofolate reductase (DHFR) or inhibit thymidylate synthetase should be tested against L1210/0 and L1210/6-MP for comparative MTX-like activity and also against one or more of a spectrum of solid tumors, eg, colon adenocarcinomas 10, 12, 36, and/or 38 as well as ovarian M5076, all of which are markedly responsive to the new triazine antifol NSC-127755 (14) and more sensitive to Baker's antifol (NSC-139105), DDMP (NSC-19494), and/or 5-MeTHHF (NSC-139490) than to MTX.[6]

The broad-based CS of P388/ara-C to a number of inhibitors of de novo purine or pyrimidine synthesis is also shown in table 6. These drugs might be considered for use in treating patients bearing tumors that initial-

[6]Unpublished data from Southern Research Institute.

TABLE 7.—In vivo tumor cell kill with PALA (optimum response, treatment every day, Days 1-9, at ≤ LD10 doses*)

Tumor	Implant size, ip	Tumor stem cells present at end of treatment	Log $_{10}$ change† in tumor cell population under treatment with PALA
P388/0	10^6	2×10^8	+2
	10^6	9×10^8	+3
	10^6	9×10^8	+3
P388/ara-C	10^6	3×10^1	-4
	10^6	2×10^4	-2
	10^6	5×10^3	-2
	10^6	1×10^3	-3
	10^6	1×10^4	-2
	10^6	2×10^3	-3
	10^6	2×10^4	-2
	10^6	5×10^2	-3

*The LD_{10} dose of PALA, ip, daily, Days 1-9, is approximately 200 mg/kg/dose.

†Log_{10} change = net log_{10} change in tumor stem cell population at the end of treatment as compared to the start of drug treatment; eg, a -3 log change means that there was a 99.9% reduction and a +3 log change means that there was a 1000-fold increase in tumor burden at the end of treatment.

ly responded to ara-C but are failing during continuing treatment, presumably due to overgrowth of ara-C-resistant cells, or for use in combination with ara-C to attempt to control the ara-C-resistant tumor cells as they appear.

Data in table 6 indicate that the body burden of L1210/0 stem cells increases by about 2 orders of magnitude under treatment up to dose-limiting toxicity with PALA, but the body burden of P388/ara-C stem cells is reduced by about 3 orders of magnitude under the same treatment with PALA. Data in table 7 show the consistent reproducibility of the CS of P388/ara-C to PALA. It should be pointed out and always remembered that such consistent and reproducible biologic responses can be accomplished only by diligent control of all variables (tumor, host mice, drug preparation, and most importantly, proper data evaluation). We have tested ara-C plus PALA against body burdens of P388/0 stem cells in excess (about 10^8 P388/0 stem cells) of the curative potential (because of overgrowth of P388/ara-C) of ara-C when used alone. Marked therapeutic synergism, probably due to control of P388/ara-C that is CS to PALA, was repeatedly observed (3), thus establishing the validity of the thesis that CS to other drugs can be therapeutically exploited, at least in murine tumor systems, if drug treatment failure may be due to overgrowth of drug-resistant tumor cells that are CS to the second drug.

There are two other important points based on the data in table 6 that deserve serious consideration:

1. Usually drugs that are inactive (do not reduce the body burden of tumor stem cells when used alone) are not considered for inclusion in drug treatment protocols. PALA, pyrazofurin, L-alanosine, and perhaps acivicin might not be considered for clinical trial because the body burden of stem cells of P388/0, generally considered to be highly sensitive to most clinically useful anticancer drugs, increases markedly under treatment up to dose-limiting toxicity with all of these drugs except acivicin. Clearly, their marked cytotoxic activity against ara-C-resistant tumor stem cells should make them prime candidates for inclusion in drug combinations being considered for clinical trial if ara-C is included in the drug combination and large body burdens of tumor stem cells (likely to contain ara-C-resistant cells) are present at start of treatment.

2. Since very sensitive, but consistent and reproducible, tumor systems are needed for detection of candidate antitumor drugs, particularly in screening for new drugs, serious consideration should be given to substituting P388/ara-C for P388/0 as the primary screen, or as one of the primary screens, if several are used. If net reduction of the body burden of tumor stem cells is the requirement for antitumor activity (as it should be), the P388/ara-C would easily detect L-alanosine, 3-deazauridine, PALA, pyrazofurin, and perhaps numerous other drugs that are now either discarded by the screen or considered to be of marginal interest because of relative insensitivity of the tumors, including P388, currently being used to screen for new agents.

Pleiotropic Phenotypic Drug Resistance

Variable cross-resistance patterns are seen among tumor cells selected for resistance to large polycyclic anticancer drugs that have markedly different biologic inhibitory activities and chemical structures, among them some drugs that bind to or intercalate with DNA, some inhibitors of mitosis, and others that inhibit protein synthesis. The concept that resistance to multiple drugs may

result from a single pleiotropic mutation to multiple drug resistance, with all of its negative implications to controlling overgrowth of drug-resistant cells in large body burdens of tumor stem cells by combination chemotherapy, given either simultaneously or sequentially, has been reported by Ling (15,16) and included in these proceedings by Ling, Biedler, and others.

Appearance of tumor cells that are resistant to a number of anticancer drugs with markedly different chemical structures and likely different biologic mechanisms of action during in vivo treatment with a single drug has been repeatedly seen by us (2), and others (6,17,18). The drugs involved are primarily large polycyclic compounds known or presumed to bind to or intercalate with DNA, to bind to tubulin or otherwise inhibit mitosis, or to inhibit protein synthesis. However, the variability of the cross-resistance patterns among these drugs is such that no prospective prediction that resistance to one of these drugs confers resistance to others among this group of compounds can be made with great confidence of accuracy, in the absence of objective data.

Murine Leukemias

Table 8 shows some of the cross-resistances that we have seen in therapy trials with P388 cells selected for resistance to some of these large polycyclic anticancer drugs. While cross-resistance among these drugs is common, obvious exceptions are also common; eg, P388/ADR shows the greatest consistency of cross-resistance to other large polycyclic drugs, but the P388/Act D retains essentially full sensitivity to ADR, dihydroxyanthracenedione (mitoxantrone), and VP-16-213. P388/VCR retains marked sensitivity to ADR, dihydroxyanthracenedione, Act D, and VP-16-213; however, P388/VCR and P388/ADR both are markedly cross-resistant to homoharringtonine, while P388/ara-C shows marked CS to homoharringtonine (19) (table 6).

Mammary Adenocarcinoma 16/C

We have recently observed resistance and cross-resistance to members of this group of large polycyclic anticancer drugs with at least one drug-sensitive solid tumor, suggesting that spontaneous pleiotropic mutation to multiple drug resistance (ADR and VCR) also may occur in advanced solid tumors. These data are shown in figures 1–3. B6C3F$_1$ (C57BL/6 ♀ × C3H ♂) mice bearing sc implanted mammary adenocarcinoma 16/C ranging in size from 50 to 500 mg (mean size, about 280 mg) were treated with ADR alone or CPA + ADR + 5-FU (CAF). Optimal regression responses of individual tumors are shown in figure 1. Overgrowth of presumed ADR-resistant tumors occurred early during treatment with ADR alone (fig 1, panel 2) and later with CAF (fig 1, panel 3). Tumor 8 (fig 1, panel 3), 288 mg at start of CAF treatment, regressed to below palpable size by Day 21 (11 days after start of CAF treatment and 4 days after second treatment) and grossly evident tumor reappeared on Day 38 after implant and 7 days before last treatment. On Day 71 after implant (26 days after last treatment), tumor-bearing Mouse 8 was killed and the tumor was passed to

TABLE 8.—Sensitivity, resistance, and cross-resistance of P388/0 leukemia and sublines selected for resistance to ADR, Act D, VCR, or *m*-AMSA and of a colchicine-resistant subline of Chinese hamster ovary (CHO) cells to treatment with a variety of polycyclic anticancer drugs (DNA binders, mitotic inhibitors, and inhibitors of protein synthesis)

		Log_{10} change* in body burden of tumor stem cells after optimal (≤ LD10) drug treatment				CHO cells
Drug	NSC No.	P388/0	P388/ADR	P388/Act D	P388/VCR	CH^RC5/ colchicine†
DNA binders or intercalators						
ADR	23127	-6	-2	-5	-5	CR
Daunorubicin	82151	-6	-1‡		-0	CR
Anthracenedione	279836	-7	-1	-6	-6	
m-AMSA	249992	-6	+2	-2‡	+2‡	
Act D	3053	-5	-1	-2	-5	
Mitotic inhibitors						
VCR	67574	-6	+3	+3	+2	
Vinblastine	49842	-3			0	CR
VP-16-213	141540	-7	+1	-5‡	-6	
Maytansine	153858	-6‡	-2‡			
Colchicine	757					R
Protein inhibitors						
Homoharringtonine	141633	-2	+2		+1	
Bruceantin	165563	-2	+1‡			

*Log_{10} change = net log_{10} change in tumor stem cell population at the end of treatment as compared to the start of treatment; eg, a -3 log change means that there was a 99.9% reduction and a +3 log change means that there was a 1000-fold increase in tumor burden at the end of treatment.

†In vitro: CR = cross-resistant and R = resistant (ref 16).

‡Single experiment.

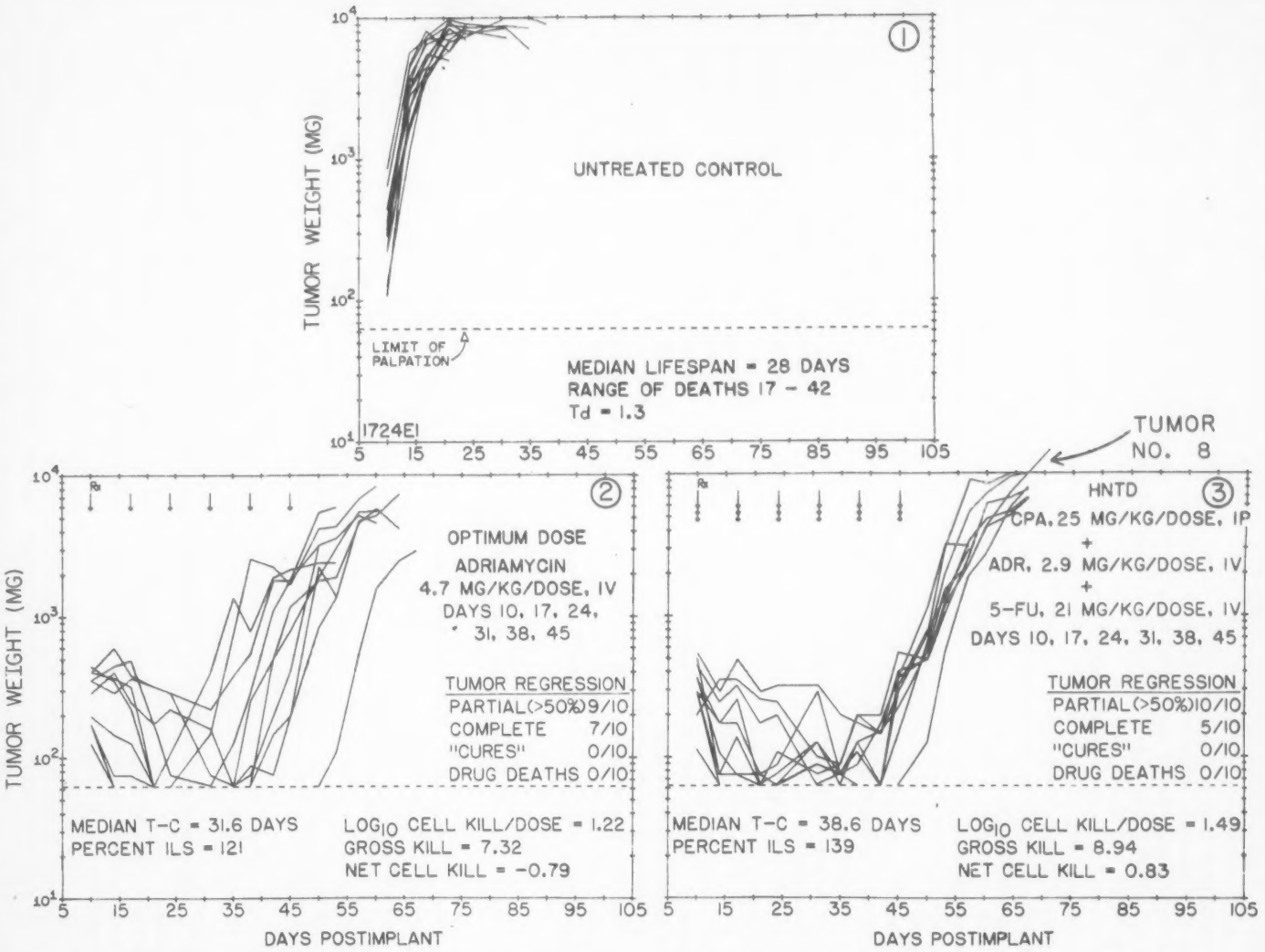

FIGURE 1.—Individual tumor response of sc implanted mammary adenocarcinoma 16/C in $B6C3F_1$ mice during treatment with ADR alone or with CPA + ADR + 5-FU. *Panel 1*—untreated control. *Panel 2*—treated with ADR, q7d × 6, starting on Day 10 postimplant. *Panel 3*—treated with CPA + ADR + 5-FU, q7d × 6, starting on Day 10 after implant. HNTD = Highest nontoxic dose from a dose-response study included as part of this experiment. Individual tumor weights were plotted until death from tumor (except No. 8).

$B6C3F_1$ mice and serially transplanted through six additional passages without drug treatment. The seventh passage in $B6C3F_1$ mice was treated in parallel (in separate mice) with the parent tumor (not previously treated with drug). The drug-resistant tumor selected by CAF (fig 1, panel 3, Tumor 8) was resistant to ADR but showed little or no resistance to either CPA or 5-FU (data not shown).

When $B6C3F_1$ mice were implanted sc bilaterally[7] and treated with ADR, the ADR-sensitive tumor failed to appear (fig 2, panel 3) before the ADR-resistant tumor grew to a lethal body burden (fig 2, panel 4). At a lower drug dose of ADR, some of the ADR-sensitive tumors grew (fig 2, panel 5) but all of the ADR-resistant tumors grew to visible size and overgrew the ADR-sensitive tumors in the same host mice (fig 2, panel 6). In a separate experiment, the same ADR-resistant 16/C and ADR-sensitive parent tumors were similarly bilaterally implanted in $B6C3F_1$ mice and treated with VCR (fig 3). The ADR-resistant tumor overgrew and killed the mice (fig 3, panels 4 and 6), while the ADR-sensitive tumor was markedly inhibited by treatment with VCR. We interpret these data to indicate that spontaneous mutation to resistance to ADR is accompanied by marked resistance to VCR in a tumor subline that had never been previously exposed to VCR. This is a convincing example of likely pleiotropic multiple drug resistance in an advanced murine mammary tumor that had never been exposed to one of the drugs (VCR) to which it is now resistant.

To our knowledge, cross-resistance of this kind has been observed and reported only once before. Kaye and Boden (20) have reported that Ridgway osteogenic sarcoma, selected for resistance to Act D under treatment with Act D, was cross-resistant to both ADR and VCR but not to CPA. Therefore, this type of pleiotropic drug resistance is obviously not unique to murine leukemias or mammary adenocarcinoma 16/C and we should expect to see it again.

Unilateral Cross-Resistance

We have previously reported that L1210/L-PAM and P388/L-PAM show marked cross-resistance to treatment with DDPt, but L1210/DDPt and P388/DDPt retain about the same sensitivity to treatment with L-PAM as do the parent L1210/0 and P388/0 (21) (tables 4 and 5). Connors (22) had previously reported that a line of Walker 256 carcinosarcoma in rats, selected for resistance to L-PAM, was completely cross-resistant to treatment with DDPt, but whether or not Walker 256 resistant to DDPt will respond to treatment with L-PAM has not been reported.

Another example of unilateral cross-resistance is shown in table 5. P388/L-PAM is cross-resistant to treatment with VCR, but P388/VCR retains full sensitivity to treatment with L-PAM.

These unilateral cross-resistances are not understood, but they could be important in drug selection for phase I-II trials or combination chemotherapy studies.

Use of Drug-Resistant Tumor Cells in New Drug Development

The 1-deaza-7, 8-dihydropteridines (listed under mitotic inhibitors in table 5: NSC-181928, NSC-269416, and NSC-330770) are of great interest because of their antitumor activity against P388/0, P388/VCR, and P388/MTX. The first compound in this series was prepared as an intermediate in the synthesis of 1-deaza-MTX (23). It was highly cytotoxic against KB cells in culture, but had very limited and equivocal cytostatic activity against L1210 in vivo. It was less active than MTX in inhibiting DHFR. NSC-181928, NSC-269416, and NSC-330770 were then prepared on the basis of their in vitro cytotoxic activity. They did not inhibit DHFR and their in vitro cytotoxicity was not reversed by folinic acid (24). They were observed to reduce the body burden of P388 stem cells by 4-5 $logs_{10}$. In studying their mechanism(s) of action, they were found to compete with colchicine for its binding sites on tubulin,[8] and VCR-resistant P388 cells showed marked sensitivity to them. Additionally, they are markedly active against P388/MTX in mice. Because of these activities against P388/0, P388/MTX, and P388/VCR, the following indications for testing in man appear plausible: (*a*) trial against MTX-sensitive tumors, particularly if resistance to MTX may be involved in ultimate treatment failure with MTX against initially MTX-sensitive tumors; (*b*) trial in combination with VCR, eg, against choriocarcinoma where MTX is useful and often curative when used alone, but where vinca alkaloids increase the therapeutic effectiveness of drug treatment when used with MTX or as second-generation treatment; and (*c*) trial in childhood ALL where both VCR and MTX are used in remission induction and maintenance therapy, and where, because of the size of the body burden of tumor stem cells at start of treatment, the overgrowth of tumor stem cells resistant to either VCR or MTX or both may be expected. Activity against both MTX- and VCR-resistant tumor cells appears to be unique, particularly with this type of structural and functional class of drugs.

DISCUSSION

Drug-resistant sublines of transplantable murine leukemias and solid tumors, with which significant and therapeutically useful reduction of the body burden of tumor stem cells can be obtained by treatment with ⩽ LD10 dos-

[7]The drug-resistant tumor was implanted sc on the right lateral thorax and the parent drug-sensitive tumor was implanted sc on the left lateral thorax of each mouse.

[8]G. P. Wheeler, personal communication.

es of representatives of all of the major chemical and functional classes of clinically useful anticancer drugs, have been isolated. Resistance, cross-resistance, and collateral sensitivity of the drug-resistant murine leukemias have been determined. Certain patterns of drug responses of these tumors appear to be consistent, and these should be useful in providing objective indications for patient selection for phase I-II clinical trials. They could also serve as guides for drug selection for clinical chemotherapy protocols where failure of drug treatment due to likely overgrowth of drug-resistant cells in initially drug-responsive tumors has occurred.

With the exception of a group of large polycyclic anticancer agents with which pleiotropic drug resistance is known to occur, mutations to drug resistance within individual chemical and functional classes of anticancer drugs are characterized by cross-resistance to very closely related drugs, particularly if the relationship is func-

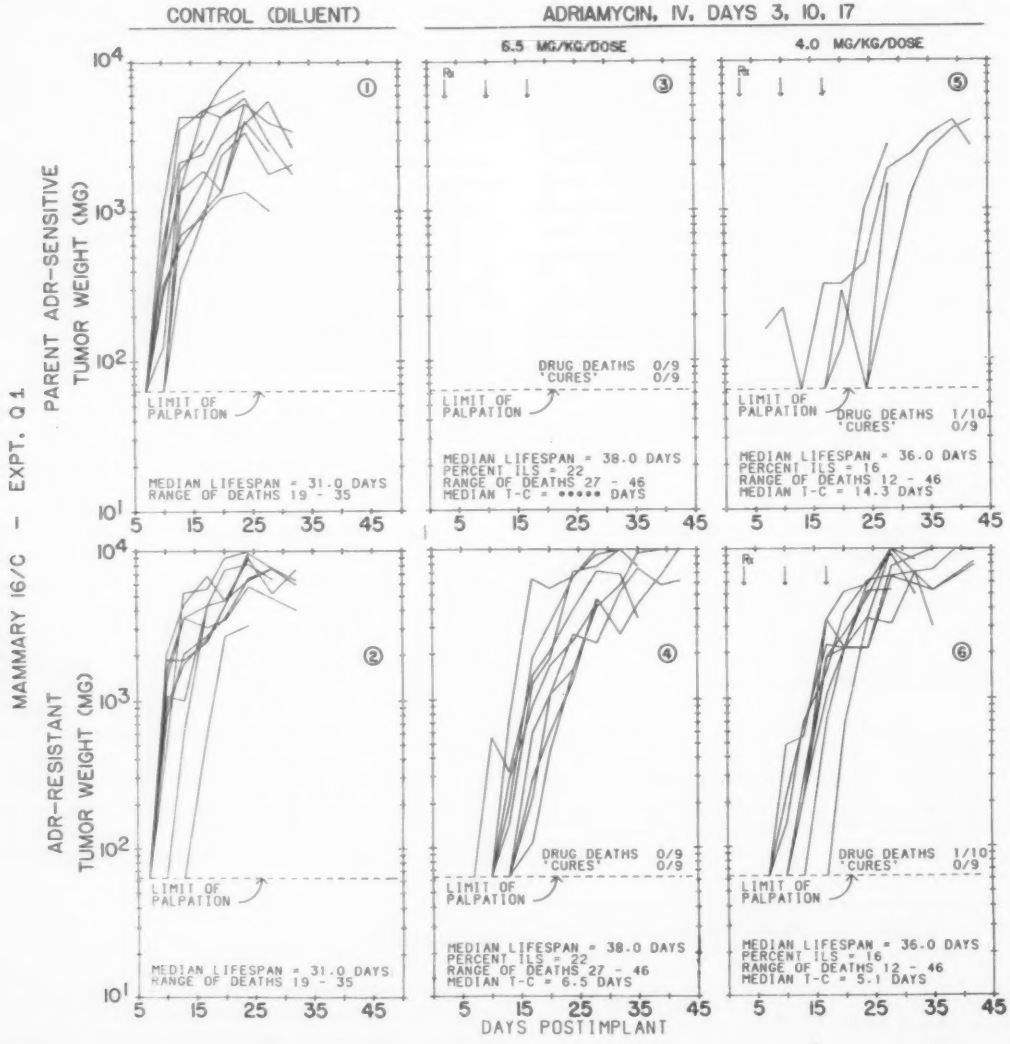
CONTROL (DILUENT)
ADRIAMYCIN, IV, DAYS 3, 10, 17
6.5 MG/KG/DOSE
4.0 MG/KG/DOSE
MAMMARY 16/C - EXPT. Q 1.
PARENT ADR-SENSITIVE TUMOR WEIGHT (MG)
ADR-RESISTANT TUMOR WEIGHT (MG)
LIMIT OF PALPATION
MEDIAN LIFESPAN = 31.0 DAYS
RANGE OF DEATHS 19 - 35
DRUG DEATHS 0/9
'CURES' 0/9
MEDIAN LIFESPAN = 38.0 DAYS
PERCENT ILS = 22
RANGE OF DEATHS 27 - 46
MEDIAN T-C = ••••• DAYS
MEDIAN T-C = 6.5 DAYS
DRUG DEATHS 1/10
'CURES' 0/9
MEDIAN LIFESPAN = 36.0 DAYS
PERCENT ILS = 16
RANGE OF DEATHS 12 - 46
MEDIAN T-C = 14.3 DAYS
MEDIAN T-C = 5.1 DAYS
DAYS POSTIMPLANT

FIGURE 2.—Individual tumor responses of bilaterally implanted mammary adenocarcinoma 16/C (parent ADR-sensitive, panels 3 and 5; and ADR-resistant subline, panels 4 and 6) in $B6C3F_1$ mice during and following treatment with ADR, q7d × 3, starting 3 days after implant. Untreated control tumors plotted in panels 1 and 2. Individual tumor weights were plotted until death from tumor.

tional and not structural, although many, often not understood, exceptions to this do occur.

Within general functional classes of anticancer agents, eg, alkylating agents, cross-resistance is closely related to the presence or absence of similar or identical functional moieties (5).

In addition to the obvious promise that utilization of data from resistance, cross-resistance, and CS studies with drug-resistant murine tumors may aid in improving drug selection for treatment of cancer patients, the promise of drug-resistant tumor cells to serve as laboratory tools for increasing our understanding of both biologic and biochemical mechanism of action of anticancer drugs as well as the mechanisms of resistance, cross-resistance, and CS is obvious and already well-established.

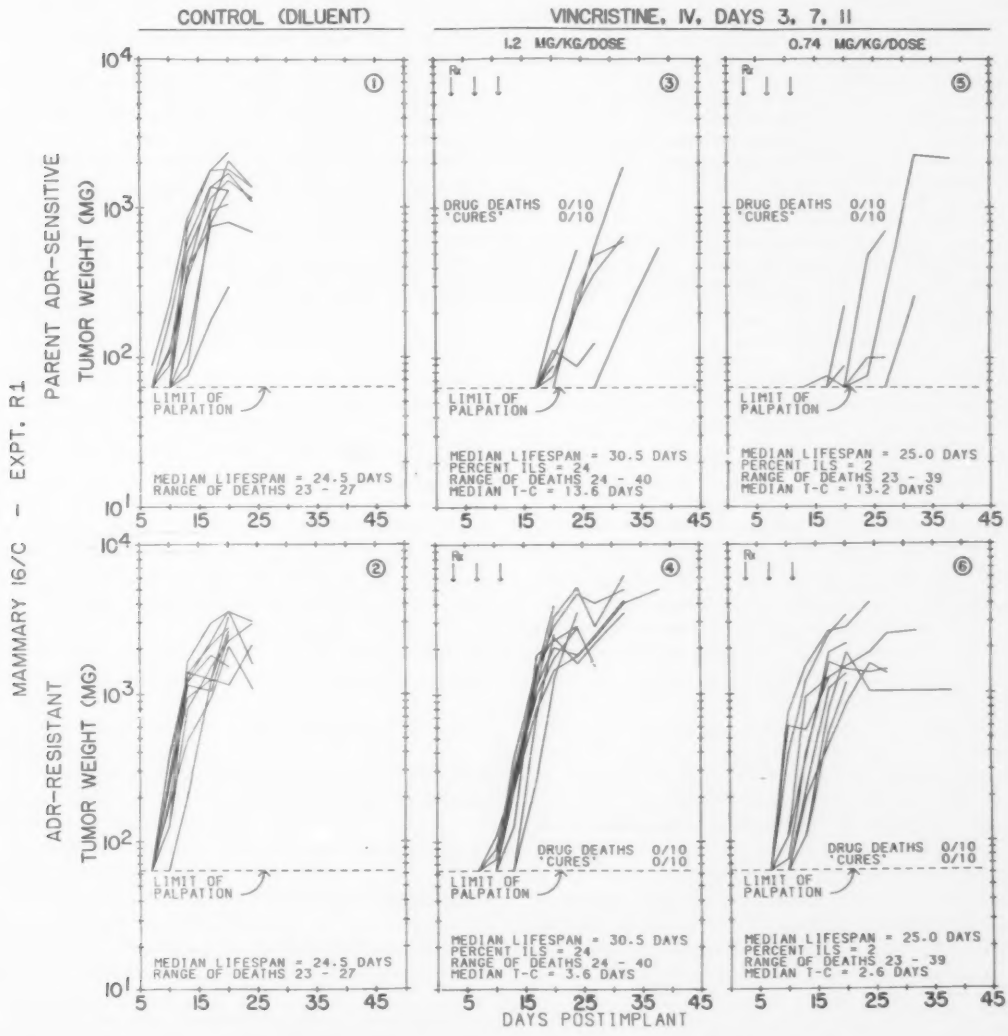

FIGURE 3.—Individual tumor responses of bilaterally implanted mammary adenocarcinoma 16/C (parent ADR-sensitive, panels 3 and 5, and ADR-resistant, panels 4 and 6) in $B6C3F_1$ mice during and following treatment with VCR, q7d × 3, starting 3 days after implant. Untreated control tumors plotted in panels 1 and 2. Individual tumor weights were plotted until death from tumor.

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Quantitative Model for Multiple Levels of Drug Resistance in Clinical Tumors

J. H. Goldie[1,2,*] and A. J. Coldman[1,3]

A mathematical model to examine the effect of cellular differentiation on phenotypic drug resistance in neoplasms is proposed. Attention is restricted to the maintenance of stem cell proliferative capacity. For fixed mutation rates, tumors in which loss of stem cell capacity occurs with high frequency will have a higher proportion of resistant stem cells than those in which such loss is infrequent. The acquisition of multiple levels of drug resistance will proceed at a disproportionately accelerated pace, which leads to greater degrees of incurability for tumors with a stem cell compartment of a given size. This model directly predicts that the phenotypic heterogeneity of slow-growing advanced clinical tumors will be very great. However, as this heterogeneity depends on both the size and the age of the tumor, there will be a period early in the development of the tumor when it should be susceptible to drug-induced cure. This potential for curability at an early stage may not be reflected in the pattern of drug responsiveness of the advanced tumor. [Cancer Treat Rep 67:923–931, 1983]

Despite significant progress in the chemotherapeutic management of a number of clinical malignancies, there are still many types of human tumors that display high orders of resistance to currently available drug treatment programs. In previous reports, we have described a quantitative and computer-based model of the phenomenon of spontaneous mutations to phenotypic resistance and how this effect can influence the drug sensitivity and curability of neoplastic cell populations (1,2). In this report, we will describe a modification of the initial minimal model that we feel more closely approximates the behavior of real systems and which may provide some insight into the processes that result in extreme levels of drug resistance.

The basic mathematical model incorporated the following assumptions:

(*a*) that cells which are stably resistant to a chemotherapeutic agent, at any specified dose, arise as a consequence of random mutations;

(*b*) that such resistant cells have the same growth kinetics as the drug-sensitive tumor cells;

(*c*) that all tumor cells are capable of unlimited proliferation, ie, they are stem cells.

These assumptions led to the notion of the nonexistence of resistant cells as a minimal condition for cure. The probability that no such cells exist was referred to as the "probability of cure" and was found to be inversely related to tumor burden.

There are a number of lines of evidence that strongly suggest that most spontaneous tumors of man (and animals) are composed of cells with a variable capacity for proliferation and self-renewal. Information has been derived from the following: morphologic examination of clinical neoplasms, cell kinetic data from human tumors (3), direct bioassay of spontaneous murine lymphomas (4), and most recently, data on the in vitro cloning of human neoplastic cells (5,6). All of these data are consistent with a model of clinical neoplasms, in which only a small fraction of the total of morphologically malignant cells in the tumor are capable of extensive proliferation and self-renewal (7). This is in contrast to a number of tumor cell lines which are maintained in tissue culture or propagated in suitable animal hosts (8). In these laboratory systems, most of the component cells appear to have clonogenic (or stem cell) capacity, but this appears unlikely to be true for most clinical neoplasms. We propose here to remove the third assumption used in modeling spontaneous resistance and to examine the effect of a stem cell compartment in which a proportion of cells randomly lose the capacity for unlimited growth (self-renewal).

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Stochastic models of tumor growth have been proposed in which stem cells divide to form either two new stem cells or two cells which have lost stem cell capacity (9–13). Considering the tumor compartment composed only of stem cells, these events would be viewed respectively as the addition (or birth) of a stem cell and the loss (or death) of a stem cell. In probability theory, such phenomena are known as birth and death processes. Characterization of such a growth process requires the specification of two rates: b(t), the birth rate, and d(t), the death rate. This framework is very flexible and can be used to produce a variety of average growth curves, such as the Gompertzian or exponential. It is important to note that the growth relationships developed relate only to the growth of the stem cell compartment and need not precisely reflect overall tumor growth, although simple assumptions about the behavior of differentiated cells do lead to a direct relationship between stem cell growth and total tumor growth (13). However, one should bear in mind that non-stem cells may continue to have limited proliferative capacity and to persist within the tumor for extended periods. In this proposed model, the term "stem cell" has a specific meaning: a single cell which can (but not necessarily does), with a certain positive probability grow to a tumor of clinically detectable size. Therefore, a non-stem cell is one that *cannot* give rise to a tumor of clinical dimensions, and will be referred to as a differentiated cell. This model will result in a system in which the clinical doubling time will, and can substantially, exceed the intermitotic times of the stem cells, and in which the cloning efficiency will be $< 100\%$.

This system of growth modeling, while flexible, does involve some computational difficulties. To simplify matters, we will assume a birth and death process in which the birth and death rates b and d are constant. The average growth rate of the stem cells will then be exponential. In general, such a system will behave as follows.

(*a*) At first, growth is very variable, as births or deaths of single stem cells greatly influence subsequent growth.

(*b*) If at any time deaths outnumber births, then the stem cell compartment, and subsequently the tumor, becomes extinct.

(*c*) Once the stem cell compartment becomes sufficiently large, its growth becomes more regular and is approximately exponential.

For simplicity, we will choose $b = 1$ and require that $d < 1$, so that births are more likely than deaths. Under these conditions, the probability that a single stem cell will give rise to a clinically detectable tumor is 1-d, and the average size of the stem cell compartment N(t) at time t is

$$N(t) = \frac{1}{1-d}\left[\exp((1-d)t) - d\exp(-(1-d)t)\right]$$

which, for a mature tumor, represents a doubling time of $\left(\frac{\ln 2}{1-d}\right)$. As can be seen, the stem cell compartment is now taking $\left(\frac{1}{1-d}\right)$ times as long to double as it would have in the absence of the process of differentiation. The greater the value of d, the greater the tumor doubling time.

Given this basic model of stem cell growth, we can now synthesize this model with the model of mutation to resistance previously proposed (1,2). An outline of the mathematics involved is given in the Appendix. This synthesis gives the following relationship between the expected number of resistant cells, E(R(t)), and time:

$$E(R(t)) = a\left[\left(t - \frac{d}{2(1-d)}\right)N(t) + \left(t + \tfrac{1}{2}\right)\frac{de}{(1-d)}^{-(1-d)t}\right]$$

In the case where $d = 0$, the above formula reduces to that we have previously described (1). Figure 1 plots the number of resistant cells by stem cell compartment size for various values of d. From this relationship, it may be seen that comparatively large values of d (ie, close to 1) lead to larger fractions of the stem cell compartment being resistant. This illustrates a very important point: it is not the number of cells but the lineage (the reproductive history) which governs the acquisition of resistance by such cells. This phenomenon is illustrated in figure 2, where it may be seen that long lineages lead to comparatively greater degrees of resistance.

Previously we have attached much importance to the probability that no resistant stem cells exist, interpreting this as a minimal probability of cure. Now, since a single stem cell (whether resistant or not) may not lead to a viable tumor, it is necessary to "adjust" this probability to reflect this eventuality. Details of this adjustment are given in the Appendix, and the resulting probability $P^c(t)$ is given by

$$P^c(t) = \exp\left\{-a\left[N(t) + \frac{2de^{(1-d)t} - 1 - d}{1-d}\right]\right\}$$

Again, for $d = 0$, this gives the formula previously obtained (1). More interestingly, however, $P^c(t)$ changes little for various values of d, implying that the enhanced acquisition of mutations in a stem cell compartment with high rates of cellular differentiation is balanced by the possibility that such cells will ultimately become extinct.

As we have done previously (2), we will now examine the situation when two agents, say, A_1 and A_2, are available for the therapy of the tumor. Using the same methods (see Appendix), it is then possible to show that $P^c_{12}(t)$ (probability that no doubly resistant cells persist indefinitely) is approximately given by

$$P^c_{12}(t) = \exp\left\{-\left(\frac{a^1}{(1-d)}\right)\left[te^{(1-d)t} - (e^{(1-d)t} - 1)\left(\frac{2+d}{2(1-d)} - \frac{1}{d}\ln(1-d)\right)\right.\right.$$

$$\left.\left. - \frac{d}{2(1-d)}(e^{-(1-d)t} - 1)\right]\right\}$$

where a^1 is a function of the individual mutation rates and is defined in the Appendix. $P^c_{12}(t)$ is plotted in figure 3 for $d = 0$ and $d = 0.98$. The form of $P^c_{12}(t)$ shows that increasing values of d lead to the development of incurability (no persistent doubly resistant stem cells) at pro-

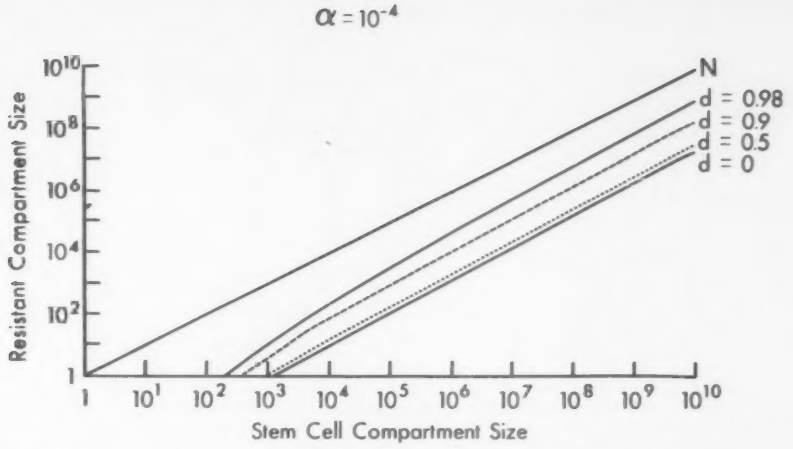

FIGURE 1.—No. of resistant stem cells plotted against the total No. of stem cells for a fixed mutation rate of $\alpha = 10^{-4}$. It may be seen that the No. of resistant cells increases directly with the No. of stem cells and with increasing death rate d. 4 values of d are used, 0, 0.5, 0.9, and 0.98, which correspond respectively to doubling times (of the stem cell compartment) of 1, 2, 10, and 50 times the generation time of the stem cells. The stem cell compartment size, N, is plotted for reference.

gressively smaller stem cell compartment sizes. The expected number of doubly resistant cells is given by

$$E[R_{12}(t)] = \frac{(\alpha^1)}{1-d}\left[\frac{t^2}{2}e^{(1-d)t}\right.$$

$$\left.-te^{(1-d)t}\left(\frac{d}{2(1-d)} - \frac{1}{d}\ln(1-d)\right) + \frac{d}{[2(1-d)]^2}\left(e^{(1-d)t} - e^{-(1-d)t}\right)\right]$$

As in the case of single resistance, when d = 0 the preceding equation reduces to the form previously found for double resistance (2). The 100% stem cell model is thus a special case of a more general process describing the acquisition of drug resistance by growing populations. However, unlike the situation where a single-step resistance phenomenon is assumed, the acquisition of double levels of resistance is no longer balanced by the probability of eventual extinction of these cells. Doubly resistant cells will accumulate at a greater rate than can be offset by their likelihood of differentiating out of the stem cell compartment of the tumor. This will greatly magnify the measured fraction of doubly resistant cells in the tumor, as compared with a cell system in which no such cell loss occurs. The source of this effect can be traced to the fact

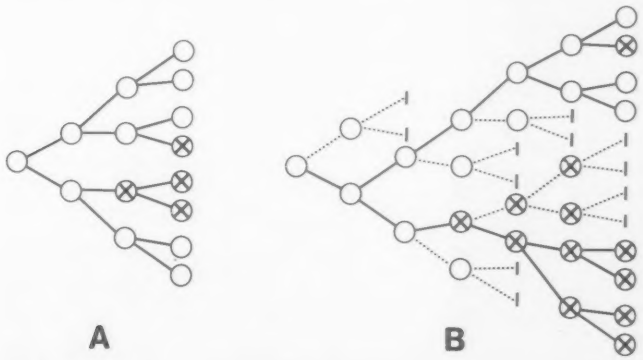

FIGURE 2.—"Growth trees" of stem cell compartments up to 8 cells for d = 0 (A) and d = 0.6 (B). A circle branching into 2 circles indicates a division of a stem cell to form 2 new stem cells, and 2 bars indicate that the division produced 2 differentiated cells. Dotted lines indicate ancestries in which all the cells have subsequently become differentiated, and solid lines indicate continued stem cell proliferation. Resistance occurs, designated by X, at the 5th branch (line connecting 2 viable stem cells) in cells which are sensitive (not resistant). All cells resulting from the division of a resistant cell are themselves resistant. In A it can be seen that this leads to 3 cells being resistant; in B, the comparatively greater number of branches leads to 5 of the 8 cells being resistant. This illustrates the enhancement of resistance caused by cellular differentiation. Note: resistance is assumed to occur deterministically rather than randomly in order to simplify illustration, but it would not be expected to do so in reality.

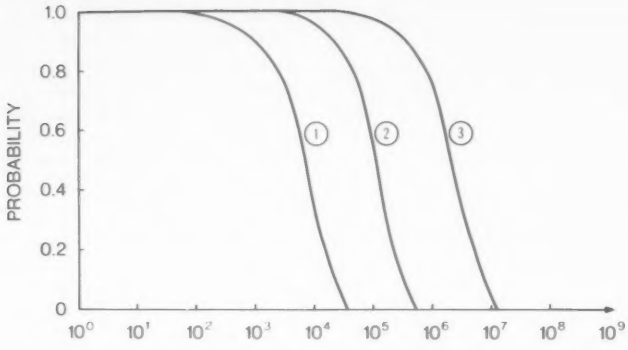

FIGURE 3.—3 curves for the probability that resistant cells do not persist to agents A_1 and A_2 that have mutation rates of $a_1 = 10^{-4}$, $a_2 = 10^{-4}$. These functions are plotted by stem cell compartment size (X-axis). (1) plots the probability that no resistant cells persist $P^0(t)$ to a single agent (ie, A_1 or A_2). This curve is approximately the same for all values of d. (3) plots the probability that no cells resistant to both A_1 and A_2 persist indefinitely for d = 0. (2) plots the probability that no cells resistant to both A_1 and A_2 persist indefinitely for d = 0.98. Note that greater values of d render the tumor stem cell compartment less curable for fixed mutation rates.

that in contrast to the single resistance case, double resistance is arising from two "birth" compartments, both of which are accumulating at a faster rate than the overall growth rate of the tumor. This net rate exceeds the rate of random cell loss, allowing the enhanced accumulation of doubly resistant cells. By inference, this phenomenon will be even more pronounced for multiply resistant phenotypes (figs 4 and 5).

Although we have used drug resistance as the phenotypic marker under consideration, this effect will generate increased heterogeneity of any cellular property arising from random, stable mutations. The quantitative effect of this process will only start to become marked for death rates > 0.9, and would therefore be a feature of slowly growing neoplasms.

DISCUSSION

If we examine the classes of advanced tumors that are presently curable by drugs, there is a significant trend towards a relationship between clinical doubling time and curability (14). Although exceptions arise both with classes of tumors and individual cases, it is generally those tumors with the shortest doubling times that are most susceptible to drug-induced cure. One could postulate that, as a group, slow-growing tumors had higher average mutation rates to resistance than fast-growing ones, but there is no direct evidence for this. Although there must unquestionably be variations in mutation rates among broad classes of tumors, it would seem reasonable to explore how other known phenomena may affect the

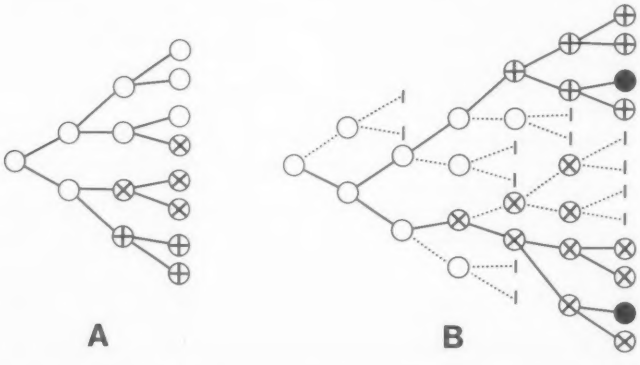

FIGURE 4.—A and B have the same growth trees as in fig 2. Here we consider a second resistance process, +, which occurs at every 6th branch, as well as X, which occurs at every 5th branch. The major change provided by this addition is the capacity to generate cells which are resistant to both agents. This is modeled by allowing each X resistant cell to become + resistant at every 6th branch, and vice versa. No doubly resistant cells emerge when d = 0 (A), but 2 are present for d = 0.6 (B) (they are indicated by a solid circle). This illustrates the enhancement of double resistance caused by cellular differentiation.

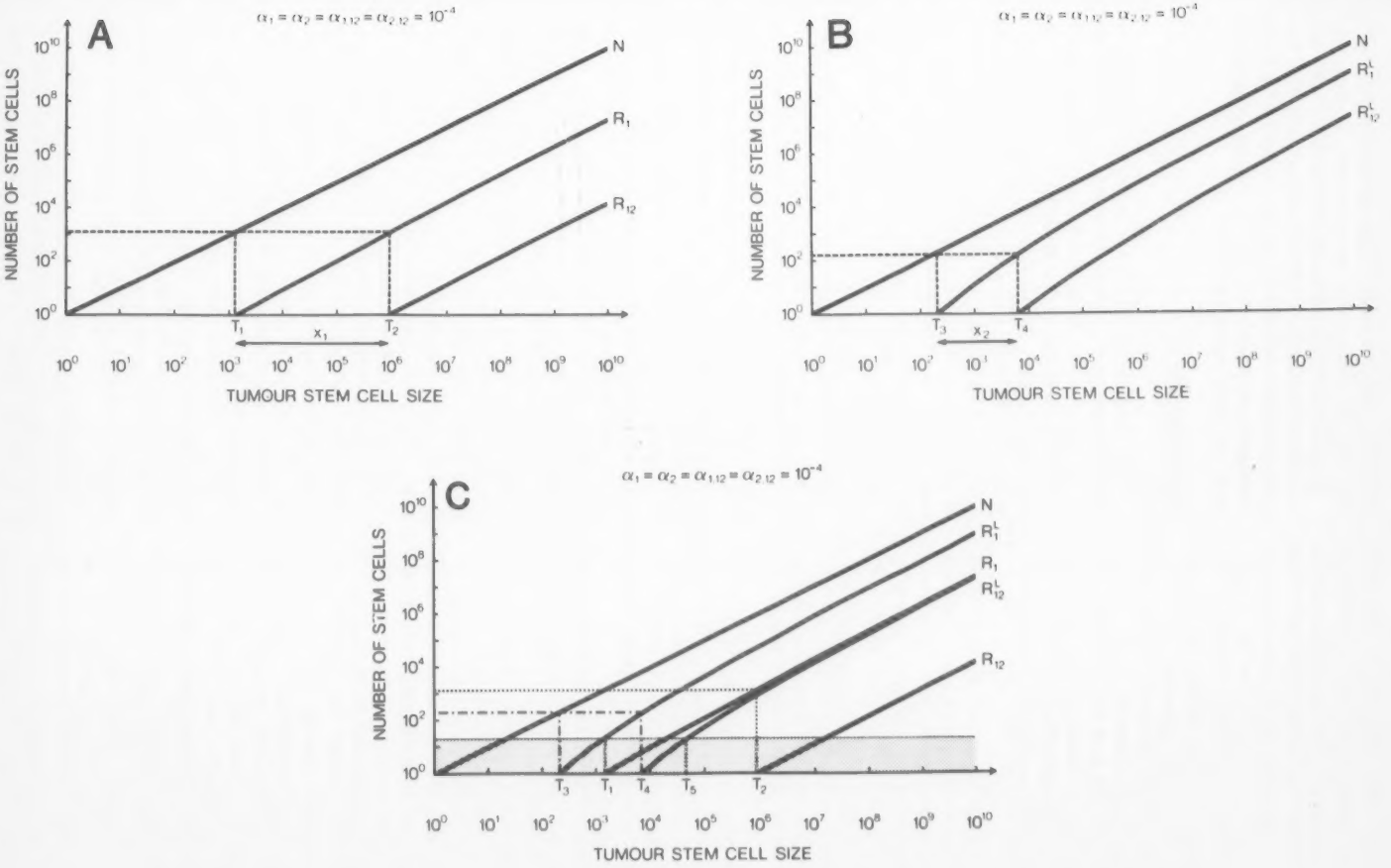

FIGURE 5.—A: the acquisition of single and double resistance (all mutation rates = 10^{-4}) when d = 0. The expected sizes of each resistant compartment are plotted. T_1 represents the size at which the expected number of singly resistant cells exceeds 1, and T_2 represents the corresponding size for doubly resistant cells. B: the same curves for a tumor in which d = 0.98. T_3 and T_4 are analogous to T_1 and T_2, respectively. C: a synthesis of the above figures with the addition of an extinction zone. Since we know (see Appendix) that the probability of cure $P^0(t)$ (for a single agent) is the same for all values of d, we may infer that the cells singly resistant (for d = 0.98) at a size less than T_1 are destined to go extinct. Thus, any resistant compartment (for d = 0.98) containing less than this No. will go extinct unless there is further growth. When the number of doubly resistant cells $R_{12}(t)$ exceeds the threshold of the extinction zone, double resistance will persist. This occurs at T_5. It can be seen from C that $T_5 < T_2$; thus, persistent resistance emerges sooner in a tumor when d = 0.98 rather than d = 0.

acquisition of resistance. The refractoriness of slow-growing tumors is often attributed to their generally low growth fraction and estimated longer intermitotic times of component cells. While these effects may modulate rates of cell kill and levels of sensitivity, it is very difficult to account for partial responses followed by relapse on this basis. Furthermore, examination of the behavior of many types of human malignancy suggest that chronic or indolent neoplasms which undergo a terminal accelerated phase of growth (ie, blast cell crisis of chronic myelogenous leukemia) are not characterized by conversion to a drug-sensitive state. It would appear that the chronicity of the growth process itself (of which long clinical doubling times are only one manifestation) functions in a manner to render the system less curable by chemotherapeutic agents.

If we assume that only a fraction of the component tumor cells retain self-renewal capacity, an assumption that seems consistent with observation, then mathematical analysis of this effect leads inescapably to the conclusion that this phenomenon on its own will have the effect of magnifying the drug resistance process. It must be stressed that no competitive advantage (ie, rapid growth, lower death rate) is given to resistant stem cells, and that this effect arises as a result of the random processes described.

While the mathematical argument predicts that the fraction of resistant cells is proportional to tumor doubling time, this is not an intuitively obvious conclusion. Simulations of growth by birth and death processes can permit a more intuitive understanding as to how this amplification of resistance arises.

If we consider the situation where, on the one hand, we have a tumor growing by a pure birth process, and contrast this to a tumor with identical mutation rates to resistance but in which there is a significant death rate (ie, $d = 0.98$), then the use of simulations makes it easier to comprehend the effect of random phenotypic change superimposed on random cell loss. If we consider a tumor of 10^9 stems cells in which no cell loss has occurred and all of the cells have clonogenic capacity, then each one of these 10^9 cells will be the product of 30 replications of their genetic material. This in turn will represent the maximum amount of ancestral diversity that can be expressed by the system after it has expanded to size 10^9. If the renewal probability is, say, 0.51 (see Appendix for definition), or equivalently a death rate of 0.98, then such a system after it has reached a size of 10^9 will display a much greater diversity and length of ancestral lineage then the pure birth model. Each of the 10^9 cells in the high death rate model will be the product of approximately 1200 genetic replications. This in turn will be a measure of the extent of genetic diversification that can arise in such a system. Furthermore, processes such as gene amplification which occur in a series of steps will be greatly augmented by the length of the ancestral lineage tree that comes about as a consequence of random cell loss.

Thus, the slow-growing system will exhibit much greater orders of phenotypic heterogeneity as compared to a system in which little or no cell loss arises. This could be one aspect or mechanism operating to generate incurability within many slow-growing tumors.

CONCLUSIONS

There appear to be two main inferences with respect to the drug treatment of advanced human tumors that can be derived from the above model. First, the broad strategic inferences that were developed from the earlier minimal models would still appear to be qualitatively correct when one goes to the more complex case. This is because, as was illustrated above, the minimal model with no loss of self-renewal can be seen simply as a special case of the more general one. Therefore, we would expect strategies previously based on this model, when $d = 0$, to remain valid (2), although the quantitative improvements from these strategies would decrease as d increases.

Second, the model does predict that the extent of heterogeneity of many slow-growing advanced human tumors may be so extreme that the chances of eradication by the use of conventional cytotoxic agents may be very small. The magnification effect operating to produce multiple levels of resistance appears to be so great as to make it highly unlikely that any realistic number of such cytotoxic agents will be adequate to eradicate all the diverse clones of cells present in the advanced tumor. However, this marked heterogeneity is related to both the size and the biologic age of the tumor. Since the shapes of the probability of cure curves are essentially unchanged for different values of d, the implication is that such markedly heterogeneous tumors will have a period early in their growth history when they should be susceptible to chemotherapeutic cure. That is, advanced tumors for which there can be no realistic expectation of cure by combinations of reasonable numbers of antineoplastic agents may well be curable when treated in the adjuvant setting.

Therefore, it is possible that the criteria for selection of agents to be tested in adjuvant trials for these groups of tumors may not be the same as those employed for more generally responsive classes of neoplasms. That is, the capacity to induce sustained complete remissions at the advanced stage may be an unrealistic criterion for drugs or drug combinations that could be expected to have an impact when used against microscopic disease. Evidence of relatively lesser degrees of biologic activity may have to suffice when developing drug protocols for use in the adjuvant treatment of these more refractory classes of tumors.

A final word may be said about the extent of heterogeneity that these advanced tumor systems will express. It is apparent from the above considerations that this

will be extreme from the point of view of agents selected on the basis of their relatively nonspecific cytotoxicity. Agents that are developed by a more specific process, directed against phenotypic properties shared by resistant cells present within the tumor, will, to a degree, overcome the problem associated with random change and reassortment of phenotypic characteristics. For example, steroid hormones or monoclonal antibodies may represent classes of agents that can, to an extent, solve the problems of heterogeneity due to random accumulation and loss of cell mutational processes.

APPENDIX

Consider a tumor with a stem cell compartment of N(t) cells at time t subject to a random process of rate α per unit time which leads to permanent drug resistance. Define N(t,s) to be the probability generating function (p.g.f.) of the process N. We will assume that N(t) is distributed as a birth and death process with constant birth rate b and death rate d. Let M(t) be the number of mutations (transitions to drug resistance) occurring in [o,t]. If we approximate N(t), by E[N(t)] then M(t,s) is given by

$$M(t,s) = \exp\{-(1-s)\int_o^t \alpha E[N(u)]du\} \qquad \ldots(1)$$

If N(0)=1 then $\lim_{t\to\infty} P\{N(t)=0\} = d/b$. We are interested only in the tumors which grow to a large size which suggest consideration of the process N^1 where $P\{N^1(t) = x\} = P\{N(t)=x \mid N(\infty)>0\}$. Then

$$N^1(t,s) = \frac{1}{1-(d/b)}\,[N(t,s) - N(t,ds/b)] \qquad \ldots(2)$$

where $$N(t,s) = \frac{d(1-s) - (d-bs)e^{-(b-d)t}}{b(1-s) - (d-bs)e^{-(b-d)t}}$$

and thus $$E[N^1(t)] = \frac{1}{(b-d)}\,[be^{(b-d)t} - de^{-(b-d)t}] \qquad \ldots(3)$$

We will now use the process N^1 for the growth of sensitive tumor stem cells.

Writing R(t) as (ref 15)

$$R(t) = \sum_{i=1}^{M(t)} X_i(t,\delta_i).$$

and letting $X_i(t,\delta_i) = X(t,\delta_i)$ for all i, then R(t) may be recognized as a filtered poisson process (ref 16), where $X(t,\delta_i)$ is the growth process of a resistant cell transformed at δ_i.

It follows then

$$R(t,s) = \exp\{ \int_0^t \alpha E[N(u)](X(t,u,s)-1)du\} \qquad ...(4)$$

We will assume that the growth of resistant cells also follows a birth and death process (rates b and d) but they are not constrained to prevent extinction. Thus

$$X(t,u,s) = N(t-u,s) \quad t \geq u$$

More precisely the p.g.f. X(t,u,s) should be determined by the birth and death process and the condition N(t) + R(t) >0 for all t (rather than the simpler condition we have imposed that N(t)>0 for all t).

Now from (2), (3) and (4) we obtain

$$R(t,s) = \left[\frac{b(1-s)-(d-bs)e^{-(b-d)t}}{b-d} \right] - \frac{\alpha b(1-s)e^{(b-d)t}}{(d-bs)(b-d)}$$

$$\left[\frac{b(1-s)-(d-bs)e^{-(b-d)t}}{(b-d)e^{-(b-d)t}} \right] \frac{\alpha d(d-bs)e^{-(b-d)t}}{(b-d)b^2(1-s)}$$

$$\exp\{ \frac{\alpha}{b(b-d)} d(1-e^{-(b-d)t})\} \qquad ...(5)$$

$$\text{Thus } E[R(t)] = \frac{\alpha}{(b-d)} \left[(bt - \frac{d}{2(b-d)})e^{(b-d)t} + \frac{de^{-(b-d)t}}{2(b-d)} \right] \qquad ...(6)$$

The probability that a resistant cell will become extinct is (d/b). Therefore the probability that resistant cells present at time t will eventually become extinct, $P^C(t)$, in the absence of any intervention is given by R(t,d/b) i.e.

$$P^C(t) = \exp\{- \frac{\alpha}{b(b-d)} (be^{(b-d)t}+de^{-(b-d)t}-(b+d))\} \qquad ...(7)$$

When two therapies are available the situation is basically similar although a little more complex. Generalizing the mutational processes in the obvious way for two therapies T_1 and T_2 i.e. $R_1(t), R_2(t), \alpha_1, \alpha_2, R_{12}(t), M_{12}(t)$ and defining $\alpha_{1,12}$ as the rate of development of resistance to T_2 in those already resistant to T_1 and analogously $\alpha_{2,12}$. Doubly resistant cells may

arise from two sources $R_1(t)$ and $R_2(t)$. Approximating $R_1(t)$, $R_2(t)$ by $E[R_1(t)]$, $E[R_2(t)]$ we have

$$M_{12}(t,s) = \exp\{-(1-s)\int_0^t (\alpha_{1,12}E(R_1(u)) + \alpha_{2,12}E(R_2(u))du\}$$

Thus $R_{12}(t,s)$ may be evaluated. $E[R_{12}(t)]$ is found to be

$$E[R_{12}(t)] = \frac{\alpha^1}{(b-d)}\left[\frac{bt}{2}^2 e^{(b-d)t} - \frac{dt}{2(b-d)}e^{(b-d)t} + \frac{de}{4(b-d)}^2 e^{(b-d)t} - \frac{de}{4(b-d)}^2 e^{-(b-d)t}\right]$$

where $\alpha^1 = \alpha_1\alpha_{1,12} + \alpha_2\alpha_{2,12}$ and $P^c_{12}(t)$ the probability that no doubly resistant cells present at t will persist indefinitely ("cure"), is given by $R_{12}(t,d/b)$, i.e.

$$P^c_{12}(t) = \exp\{-\frac{\alpha^1}{(b-d)}\left[te^{(b-d)t} - \frac{1}{2b(b-d)}(2b+d)e^{(b-d)t} - 2(b+d) + de^{-(b-d)t}\right]\}$$

If this process is cast in a discrete framework then instead of birth and death rates there is a single parameter P, the renewal probability, which specifies the probability that a stem cell divides to form two new stem cells. 1-P is then the probability that two non-stem cells are formed at division. For sustained growth to occur $P > 0.5$. P and b,d may be related since the probability that a single stem cell will divide to form two new stem cells (for the birth and death process) is $\frac{b}{b+d}$, therefore

$$P = b/(b+d).$$

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Phase I–II Study of 120-Hour Infused 5-FU and Split-Course Radiation Therapy in Localized Non-Small Cell Lung Cancer[1]

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Thirty patients with locally advanced nonresectable non-small cell bronchogenic carcinoma were studied for tolerance and response to 120-hour continuously infused 5-FU (IFU) combined with chest x-ray therapy. The IFU was escalated in patient groups from 20 to 35 mg/kg/24 hours. Radiation was also escalated in patient subsets from 200 to 400 rads/fraction using a split-course technique to a total dose of 5000 rads. Tolerance was exceeded by IFU doses of 35 mg/kg/day. The complete response rate was ten of 28 patients (36%); a complete response was associated with a significantly prolonged survival. Radiosensitization of lung cancer by IFU appears clinically feasible in man. [Cancer Treat Rep 67:933–936, 1983]

Recent clinical studies have shown that failure to control tumor growth in the chest is a significant cause of decreased survival and death in bronchogenic carcinoma (1). Patients who achieve a complete clinical response (CR) in the chest live significantly longer than other patients, irrespective of histology (2,3). In nonresectable disease, radiation is the major mode of therapy, but produces a CR in only a minority of patients (2,3). Enhanced human tumor cell killing (radiosensitization [RS]) can be achieved in vitro by combining 5-FU with x-rays, but only under conditions clinically equivalent to infused 5-FU (IFU), where the *postradiation* drug exposure is > 24 hours (4). Because 5-FU is rapidly catabolized in the liver, RS cannot be achieved with bolus 5-FU schedules (4,5). In this study, we have examined the tolerance of limited disease non-small cell lung cancer patients to IFU under conditions likely to produce RS. A phase I format was used to examine patient tolerance to this approach, using essentially full-dose chest radiation.

PATIENTS AND METHODS

Thirty patients with biopsy-proven nonresectable (limited stage III) non-small cell lung cancer were sequentially studied using the schema shown in table 1. There were three females and 27 males, with a median age of 62 years. The protocol was designed to study escalation of *both* the IFU and x-ray doses. A split-dose radiation technique was used throughout the study to permit evaluation of combined toxicity during the first treatment period *and* during the subsequent 21-day rest period (for delayed acute effects). A *total* dose of 5000 rads was used, since conventional palliative split-course techniques (4000 rads) are associated with reduced responses and survival, while a 5000 rads total dose has been shown to be equivalent to higher doses in randomized trials of x-rays alone (3). In all patients, the entire tumor, mediastinum, and ipsilateral supraclavicular region were radiated, using AP/PA portals throughout, without compensation for lung absorption, but with tissue compensators to homogenize the dose in the vertical portal limits. A PA spine block was added at 4500 rads to prevent radiation myelitis (see below). The prescribed dose in all cases was 5000 rads; the mean dose obtained was 4797 rads. All patients completing the first part of the split-course treatment (N = 28 patients or 93%) were considered evaluable for toxic effects and response.

The IFU infusions were administered using an IVAC pump beginning within 3 hours of the first x-ray treatment. The IFU was made up in 1.0 L of 5% dextrose/water and was administered (light-protected) in a constant fashion over 24 hours. This was repeated daily × 5 for a 120-hour infusion. Patients who developed grade 1 neutropenia (table 2), grade 2 thrombocytopenia, or any other form of grade 2 toxic effects *during* an infusion had a 50% daily IFU dose reduction for the remainder of the

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TABLE 1.—Schema of the trial

Patient No.	Phase I (Days 1-5)	Phase II (Days 6-27)	Phase III (Days 28-32)	Phase IV (Days 33+)
1-3	5-FU—20 mg/kg/24 hrs for 5 days; XRT* —1250 rads in 5, 250-rad daily fractions	No treatment	5-FU—20 mg/kg/24 hrs for 5 days; XRT—1000 rads in 5, 200-rad daily fractions	XRT—2750 rads in 14, 200-rad fractions given 5 fractions/wk between Days 35 and 52
4-7	5-FU—20 mg/kg/24 hrs for 5 days; XRT—1700 rads in 5, 340-rad daily fractions	No treatment	5-FU—20 mg/kg/24 hrs for 5 days; XRT—1000 rads in 5, 200-rad daily fractions	XRT—2300 rads in 12, 300-rad fractions given 5 fractions/wk between Days 35 and 50
8-12	5-FU—25 mg/kg/24 hrs for 5 days; XRT—1700 rads in 5, 340-rad daily fractions	No treatment	5-FU—25 mg/kg/24 hrs for 5 days; XRT—1000 rads in 5, 200-rad daily fractions	XRT—2300 rads in 12, 200-rad fractions given 5 fractions/wk between Days 35 and 50
13-20	5-FU—25 mg/kg/24 hrs for 5 days; XRT—2000 rads in 5, 400-rad daily fractions	No treatment	5-FU—25 mg/kg/24 hrs for 5 days; XRT—1000 rads in 5, 200-rad daily fractions	XRT—2000 rads in 10, 200-rad fractions given 5 fractions/wk between Days 35 and 46
21-25	5-FU—30 mg/kg/24 hrs for 5 days; XRT—2000 rads in 5, 400-rad daily fractions	No treatment	5-FU—30 mg/kg/24 hrs for 5 days; XRT—1000 rads in 5, 200-rad daily fractions	XRT—2000 rads in 10, 200-rad fractions given 5 fractions/wk between Days 35 and 46
26-30	5-FU—35 mg/kg/24 hrs for 5 days; XRT—2000 rads in 5, 400-rad daily fractions	No treatment	5-FU—35 mg/kg/24 hrs for 5 days; XRT—1000 rads in 5, 200-rad daily fractions	XRT—2000 rads in 10, 200-rad fractions given 5 fractions/wk between Days 35 and 46

*X-ray treatment.

infusion. Patients having greater toxic effects during (or following) an infusion had their infusion stopped (where relevant) and resumed on the next cycle at 50% of the previously toxic dose.

Patients were analyzed for response and survival. A CR was defined as disappearance of all disease by conventional roentgenographic methods (subject to postradiation fibrotic ambiguities), a partial response (PR) as 50% reduction in the product of the diameters of all measurable lesions (without new lesions), and nonresponders as less than a PR. Survival was dated from the first day of treatment. Toxicity definitions are shown in table 2.

RESULTS AND DISCUSSION

Of the 30 patients entered in the study, 28 were eligible for evaluation (two nontoxic patients moved out of the area immediately after the first treatment cycle). The most prominent side effects were gastrointestinal, usually mild (grade 1) nausea without vomiting. At low IFU doses (20-25 mg/kg), this appeared about the same as expected with x-rays alone. The more severe cases were clearly 5-FU dose-dependent, with eight of nine patients having treatment-induced vomiting at 30 and 35 mg/kg. In one case, this side effect was dose-limiting and was associated with significant dysphoria and mild cerebellar signs consistent with 5-FU CNS toxicity. These toxic effects cleared completely with 5-FU dose reduction.

The most common dose-limiting toxic effect was mucositis (stomatitis), which occurred at a dose-limiting level at the higher IFU doses (two of four [50%] patients received 35 mg/kg) similar to that reported for 120-hour infusions without added x-rays (6). In one case, this was associated with very severe (grade 4) esophagitis, indicating enhancement of the local mucositis by the esophageal radiation, which had been 2000 rads in five fractions, ie, the highest x-ray fractionation regimen. Overall (CNS + mucositis), the 35 mg/kg dose was excessive in three of four (75%) patients receiving the 120-hour IFU dose. The only other *regional* toxic effect noted was reversible moist desquamation of the skin in the supraclavicular region where the skin x-ray dose is higher (two of 28 patients). Somewhat surprisingly, radiation pneumonitis did not appear to be enhanced, since only one patient developed chest symptomatology sufficient to require hospitalization and therapy. This episode cleared completely, but left the patient with limited respiratory reserve. However, the patient returned to work on a part-time basis and lived for > 2 years, eventually dying from brain metastases without clinical recurrence in the chest. However, the use of alternative x-ray treatment plans (eg, oblique fields which were specifically avoided to reduce lung parenchymal radiation) might increase the hazard of this complication.

It is an interesting observation that hematologic toxicity was not affected by the combined treatment. An exaggerated toxic effect on the bone marrow has been suggested (7) when thoracic radiation is combined with chemotherapy. The data in table 2 show this not to be the case when true *marrow* parameters (8) are followed. We have shown elsewhere that the only significant hematologic effect of thoracic radiation is on the blood lymphocyte population (9). The marrow-sparing characteristic of IFU is not altered by the coapplication of full doses of

TABLE 2.—Dose-related toxic effects

Toxicity (grade)	No. of patients (%)			
	20 mg/kg/day of 5-FU	25 mg/kg/day of 5-FU	30 mg/kg/day of 5-FU	35 mg/kg/day of 5-FU
Hematologic				
Neutrophils				
1000–1699 cells/mm^3 (1)	0	0	0	0
750–999 cells/mm^3 (2)	0	0	0	0
⩽ 749 cells/mm^3 (3)	0	0	0	0
Platelets				
100,000–124,999 cells/mm^3 (1)	0	0	0	0
60,000–99,999 cells/mm^3 (2)	0	1 (8)	0	0
⩽ 59,999 cells/mm^3 (3)	0	0	0	0
Upper intestinal				
Nausea (1)	5 (71)	3 (25)	1 (20)	0
Nausea, mild vomiting (2)	2 (28)	2 (17)	1 (20)	1 (25)
Severe vomiting (3)	0	0	1 (20)	1 (25)
Stomatitis				
Minimal (1)	0	1 (8)	2 (40)	0
Mild dysphagia (2)	1 (14)	1 (8)	0	1 (25)
Severe dysphagia (3)	0	1 (8)	0	0
Maximal dysphagia (esophagitis) (4)	0	0	0	1 (25)
CNS	0	0	0	1 (25)
Skin (moist desquamation)	0	1 (8)	1 (20)	0
Pneumonitis (delayed)	0	1 (8)	0	0
Totals	7 (100)	12 (100)	5 (100)	4 (100)

thoracic radiation.

The overall median survival of the entire group was 290 days. Response to treatment was also determined, so far as was possible. Of the 28 evaluable patients, ten (36%) achieved a CR. As in other studies, a CR was associated with significantly prolonged median survival (590 days). This was statistically (10) better ($P < 0.001$, log rank test) than patients achieving a PR (median survival = 250 days), or patients who were nonresponders (median survival = 147 days), the survival difference between the latter two results not being significant. These results appear at least as good as conventional x-ray therapy in existing schedules.

RS using IFU appears worthy of further study. We believe it is critical at this stage to determine the shortest treatment cycle length in which IFU can be combined with x-rays under pharmacologic conditions of RS. Our laboratory studies (11) show clearly that the serum 5-FU levels achieved during infusions of this type are similar to those needed to induce both cytotoxicity of human tumor cells in culture (5) and to induce RS (4). We have therefore hypothesized (4) that higher-dose IFU coupled to x-ray hyperfractionation (more than one x-ray treatment fraction per day) may be optimal; such trials are in progress (12,13).

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Treatment of Hormone-Resistant Metastatic Carcinoma of the Prostate With 5-FU, Doxorubicin, and Mitomycin (FAM'): A Preliminary Report[1]

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We treated 19 patients who had hormone-resistant adenocarcinoma of the prostate (stage D-2) with 5-FU, doxorubicin, and mitomycin (FAM'). All patients had extremely poor prognostic factors. Of the 16 patients evaluable for response, 44% had stabilization of disease and 56% had progressive disease. The median survival time for the stable disease group was 48 weeks, whereas that for the nonresponders was 26 weeks. The difference in survival time was statistically significant ($P < 0.002$). The FAM' regimen was well-tolerated with minimal nausea, no vomiting, and moderate hematologic toxicity. [Cancer Treat Rep 67:937–939, 1983]

In 1973, the National Prostatic Cancer Project (NPCP) initiated the first chemotherapeutic trials in hormone-resistant prostate cancer (HRPC) and developed new criteria for response (1). A few single agents were identified as effective in these trials. 5-FU was found to produce a 36% objective response rate and doxorubicin (ADR), a 24% response rate (2); mitomycin remains untested. The combination of these three drugs was reported to be active in advanced HRPC (3,4). We report here on the preliminary results of a pilot study that was begun at our institution in August 1980.

PATIENTS AND METHODS

The pretreatment characteristics of our 19 patients are summarized in table 1. All patients showed evidence of disease progression at the time of initiation of chemotherapy. Adequate hepatic and renal functions were required for entry in this study.

The FAM' chemotherapy regimen consisted of 5-FU at a dose of 600 mg/m^2/24 hours (total dose not to exceed 1100 mg/24 hours) via a continuous iv infusion for 5 days on Days 1–5 and 28–32; ADR at a dose of 30 mg/m^2 by iv bolus over 15–25 minutes on Days 1 and 28 (cumulative dose not to exceed 550 mg/m^2); and mitomycin at a dose of 10 mg/m^2 by iv bolus on day 1. The cycle was repeated every 56 days. This treatment was continued until progression of disease was observed. All patients were followed until death. A minimum of two cycles of FAM' was required for a patient to be considered evaluable for response. Patients who received one cycle and demonstrated overt disease progression were considered failures. All patients were evaluable for toxic effects. Those patients who died before Day 28 of the first cycle were evaluable for toxic effects but not for response or survival. Prior to therapy and every two cycles thereafter, all patients were evaluated with cbc, SMA-18 chemistry profile, serum prostatic acid phosphatase (PAP-enzymatic method), urinalysis, bone marrow aspirate and biopsy, chest radiogram, skeletal survey, and radionuclide bone and liver scans. Because a single PAP determination by the enzymatic method is unreliable (5), at least two measurements for every change of > 50% from the initial level were made, and the mean value was considered to be the true level. The NPCP criteria for response were used. The dose was modified as follows: for a wbc count nadir of 500–1499/mm^3 and a platelet count nadir of 30,000–74,999/mm^3, 5-FU and ADR were reduced by 25%; for any lower nadir counts the above drugs were reduced by 50%; 5-FU was increased by 25% for any wbc count nadir > 3000/mm^3 or platelet count nadir > 100,000/mm^3; and previously irradiated patients had a dose reduction of 25% for all drugs. The difference between the survival curves of responders and nonre-

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TABLE 1.—Pretreatment characteristics of 19 patients with metastatic HRPC treated with FAM′

Characteristic	No. of patients
Age (yrs)	
50–59	4
60–69	11
70–79	4
Median: 62	
Eastern Cooperative Oncology Group performance status	
1 (symptoms, ambulatory)	11
2 (in bed < 50% of the time)	3
3 (in bed > 50% of the time)	4
4 (in bed 100% of the time)	1
Prior therapy	
Diethylstilbestrol	3
Orchiectomy	11
Diethylstilbestrol + orchiectomy	5
Cyclophosphamide	8
Radiation therapy*	6
Histologic differentiation	
Moderately well-differentiated	7
Poorly differentiated	12
Progressive disease detected by change in—	
Skeletal survey	18
Bone scan	19
Liver scan	4
Computerized axial tomographic scan	5†
Bone marrow infiltration	9
Serum acid phosphatase (rising)	18
Measurable soft tissue tumors	8

*> 2000 rads megavoltage radiotherapy to whole pelvis and boost dose to prostate area.

†Abdominal computerized axial tomographic scan was obtained in 8 patients.

sponders was calculated by means of the Mantel-Haenszel test (6). Since the samples to be compared were small, we also did an exact test with ties by using the Savage statistic (7).

RESULTS

The FAM′ regimen was extremely well-tolerated, without any drug-related deaths. One patient experienced an episode of fever without sepsis during nadir neutropenia. Two patients developed transient gross hematuria during nadir thrombocytopenia. Four patients (21%) experienced mild nausea; none vomited. Mild mucositis was seen in two patients (11%) and alopecia in four (21%). Sixteen patients were evaluable for response and survival. Seven of these (44%) had stabilization of the disease that lasted for a median of 38 weeks (range, 28–69). Nine patients (56%) showed progressive disease. Remarkable alleviation of bone pain was noted in nine of the 16 patients (56%). The stable disease group had a median survival time (MST) of 48 weeks (range, 37–77), whereas the MST for the progressive disease group was 26 weeks (range, 10–46). The difference in survival between the two groups was statistically significant ($P < 0.002$).

DISCUSSION

In an extensive literature review, Torti and Carter (8) summarized ten important variables that affect the response and survival in patients with metastatic adenocarcinoma of the prostate treated with hormonal therapy or chemotherapy. In our patients an average of seven of these adverse variables were present at the beginning of FAM′ treatment; in addition, eight patients had been pretreated with cyclophosphamide. The most difficult problem in evaluating the results of chemotherapy for prostate cancer is the lack of uniform, easily applicable and measurable criteria for objective response. Among the best established and most widely used criteria are those adopted by the NPCP (1). The most notable problem with them is the requirement that the PAP level become completely normal in partial response and that it decrease in stable disease. Thus, in patients lacking measurable soft tissue metastases, the normally occurring wide and unpredictable fluctuations of the PAP levels (5) can easily change a classification of response to treatment from partial response to stable disease, and vice versa, without necessarily indicating failure or success of therapy. In this study we considered the mean PAP value as the true level; thus, we reclassified two patients from the partial response to the stable disease group. A similarity in the survival times of patients with partial response and stable disease was recently reported by Ihde et al (9) and was confirmed by Slack et al (10). Considering the characteristics of our patients and the extensive staging procedures used by us, the stable disease rate of 44% represents one of the best therapeutic results reported when the NPCP criteria were applied. Recently, Logothetis et al (3), using a similar combination of ADR, mitomycin, and 5-FU (DMF) in a different scheme of administration and employing their own criteria for response, obtained an overall 45% objective response rate, with an MST of 48 weeks for the responders, which is similar to our MST with FAM′. The severe toxicity of DMF contrasts with the mild toxicity observed with FAM′. The difference is probably attributable to the continuous iv administration of 5-FU by us, which has been reported to result in drug levels that are lower in bone marrow than in plasma (11). Our results indicate that FAM′ is effective in the palliative treatment of advanced HRPC; it prolongs the life of the responders significantly and is associated with minimal toxicity.

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Administration of Gallium Nitrate by Continuous Infusion: Lack of Chronic Nephrotoxicity Confirmed by Studies of Enzymuria and β_2-Microglobulinuria[1,2]

Brian Leyland-Jones,* Ravi B. Bhalla, Fouad Farag, Linda Williams, Craig J. Coonley, and Raymond P. Warrell, Jr.[3]

Enzymuria and β_2-microglobulinuria are sensitive markers of injury to renal tubular cells. We evaluated these markers in 41 patients with advanced malignant lymphoma who received gallium nitrate administered as a continuous infusion during a recent phase I–II study. In contrast to our findings with cisplatin, we observed no significant increase in enzyme excretion or β_2-microglobulinuria in these patients even after long-term treatment. Our results indicate that gallium nitrate administered by infusion does not produce cumulative renal tubular toxicity. [Cancer Treat Rep 67:941–942, 1983]

Increased urinary excretion of β_2-microglobulin and certain enzymes, leucine aminopeptidase (LAP) and *N*-acetyl-β-glucosaminidase (NAG), is a sensitive marker of damage to renal tubular cells (1–3). Chronic renal insufficiency is associated with the use of several metal-based drugs. We recently showed that increased enzymuria and β_2-microglobulinuria are well-correlated with chronic nephrotoxicity produced by cisplatin (1). Subsequently, we performed serial analyses of these markers in patients with malignant lymphoma who received gallium nitrate in a recent phase I–II study (4). We found that gallium nitrate did not produce serious chronic nephrotoxicity when the drug was administered as a continuous infusion.

PATIENTS AND METHODS

Forty-one patients who received gallium nitrate by continuous infusion at doses of 300 or 400 mg/m^2/day × 7 were studied. Urine samples were collected at the beginning of the infusion (Day 1), on Day 4 of the infusion, and 24 hours after the completion of the infusion (Day 8). To prevent degradation of β_2-microglobulin, the urine was placed in vials containing 1 M tris-HCl (pH = 7.8) which maintained urinary pH > 5.5. The samples were then refrigerated for subsequent assay.

NAG and LAP activities were assayed by methods previously described (1). β_2-Microglobulin was assayed by radioimmunoassay using the Phadebas β_2-Micro Test kit (Pharmacia Diagnostics, Piscataway, NJ). Urinary creatinine concentration was determined using the Gemini Creatinine Reagent Kit (Electronucleonics, Fairfield, NJ). All assays were done in duplicate. Values of NAG and LAP activities and β_2-microglobulin concentration were expressed per milligram of urinary creatinine.

RESULTS

The mean values for NAG, LAP, and β_2-microglobulin excretion measured in 41 patients throughout all of their courses are shown in figure 1. We compared the measurements on Day 4 and Day 8 with baseline values (Day 1) obtained from patients during their first infusion. As shown in table 1, there was no correlation of changes in urinary excretion of enzymes or β_2-microglobulin with increases in serum creatinine. The transient rise in β_2-microglobulin observed in the first course was not sustained (fig 1). Urinary β_2-microglobulin increased < 0.05 μg/ml in six patients whose serum creatinine rose 2.0–6.8 mg/dl over baseline. In patients who received multiple courses (five courses in two patients; four courses in five patients), values were higher after the second course of gallium nitrate than during the third or fourth courses. Changes in NAG and LAP activity for each patient generally paralleled each other for each course.

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TABLE 1.—Day 4/Day 1 (D4/D1) and Day 8/Day 1 (D8/D1) ratios of NAG, LAP, and β_2-microglobulin correlated with changes in serum creatinine in 41 patients after the initial course of gallium nitrate

Serum creatinine (mg/dl)	NAG		LAP		β_2-microglobulin	
	D4/D1	D8/D1	D4/D1	D8/D1	D4/D1	D8/D1
<0.4	1.2	2.86	1.62	1.72	3.38	91.59
0.5-1.0	2.34	5.69	5.31	0.65	22.99	186.7
>1.1	0.31	1.92	-0.097	-0.44	9.35	26.09

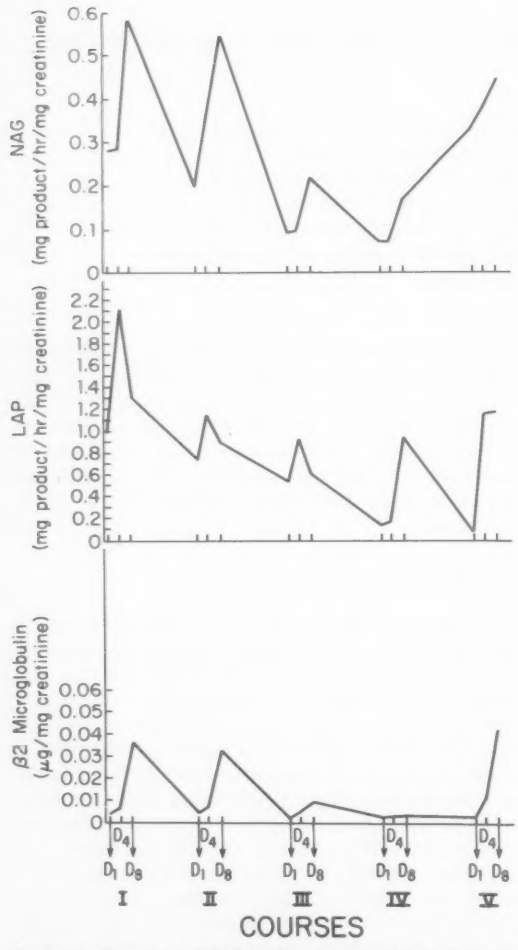

FIGURE 1.—Mean value of NAG, LAP, and β_2-microglobulin for 41 patients. D1, D4, and D8 = Day 1 (baseline), Day 4 of infusion, and Day 8 (1 day after end of infusion), respectively.

DISCUSSION

Daily doses of gallium nitrate of ≤ 300 mg/m² administered by continuous infusion infrequently produce overt renal toxicity as measured by changes in serum creatinine concentration (4). We have not observed cumulative renal toxicity in patients who received intermittent infusions for periods > 18 months (4). Our studies of enzymuria and β_2-microglobulinuria confirm this observation since these values also did not increase over time. In fact, excretion of these tubular markers was lower in later courses than in the initial infusions.

The elevation in urinary NAG and LAP activities observed here is approximately 1 log less than that seen in our previous studies with cisplatin (1). This lower degree of elevation, the absence of significant β_2-microglobulinuria, and the observation that serum creatinine did not rise with additional infusions indicate a lack of cumulative renal tubular toxicity due to gallium nitrate which is markedly different from clinical experience with cisplatin.

β_2-Microglobulin is a specific marker of renal tubular dysfunction and increased urinary excretion of this peptide is well-correlated with progressive renal disease (2,3). The lack of significant β_2-microglobulinuria in this study suggests that gallium nitrate does not produce permanent renal tubular dysfunction. Our results support the pathologic observations of Newman et al (5), who found that gallium nephrotoxicity is associated with precipitation of gallium-calcium-phosphate crystals in renal tubules. These potentially reversible lesions are distinct from those caused by cisplatin which is directly toxic to "brush border" cells (6) and which commonly produces cumulative nephrotoxicity (7).

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Phase II Trial of Mitomycin, Vinblastine, and Cisplatin (MVP) in Non-Small Cell Bronchogenic Carcinoma[1]

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A phase II trial of mitomycin, vinblastine, and cisplatin was undertaken in 26 patients with non-small cell carcinoma of the lung. A major response rate of 46% was seen in measurable and evaluable disease, with a complete response rate of 12%. Median duration of response is 6.5+ months (range, 2.5–19+). Toxic effects included moderate myelosuppression, mild neuropathy, mild azotemia, and severe nausea and vomiting. These results are similar to previously reported studies using vinca alkaloids and cisplatin alone. [Cancer Treat Rep 67:943–945, 1983]

Despite recent advances in chemotherapy, the treatment of inoperable non-small cell carcinoma of the lung remains poor. Recently, the combination of the vinca alkaloid vindesine and cisplatin has resulted in a major response rate of approximately 40%, with a median survival duration of 12 months. However, a complete response rate of only 10% was noted (1).

We have previously shown a 27% response rate in a small group of untreated patients with divided-dose vinblastine, for a median duration of 5.5 months (2). These data are remarkably similar to those for vindesine as a single agent (3). Patients with adenocarcinoma of the lung have previously shown a 25% response rate to the antitumor antibiotic mitomycin used as a single agent (4).

Thus, the combination of mitomycin, vinblastine, and cisplatin (MVP) was evaluated with the hope of increasing response rates without prohibitive toxicity.

MATERIALS AND METHODS

Twenty-six patients with histologically documented, inoperable adenocarcinoma, squamous cell carcinoma, or large cell anaplastic carcinoma of the lung were entered in this study. All patients had a performance status ≥ 60 on the Karnofsky scale. Either measurable or evaluable disease was required. Previously described response criteria were used (2). For patients with evaluable disease a response [according to the data of Eagan et al (5)] was graded as follows: *complete response*—all evaluable disease regressed; *partial response*—unequivocal shrinkage of evaluable disease; or *stable disease*—no disease progression (there were five patients in this category). Response had to be maintained for ≥ 1 month.

The treatment schedule was as follows: vinblastine, 4 mg/m^2 on Days 1 and 2 and every 2 weeks thereafter; cisplatin, 75 mg/m^2 on Days 1 and 29 and every 6 weeks thereafter; and mitomycin, 10 mg/m^2 on Day 15 and every 6 weeks thereafter.

Dose modifications for vinblastine and mitomycin were as follows: for total wbc count $< 3000/\mu l$ and platelet count $< 150{,}000/\mu l$, a 75% dose was given; for wbc count $< 2500/\mu l$ and platelet count $< 100{,}000/\mu l$, 25% was given; and for wbc count $< 1500/\mu l$ or platelet count $< 50{,}000/\mu l$, both drugs were withheld. Cisplatin was withheld if serum creatinine was > 2.0 mg/dl or if creatinine clearance fell to < 50 ml/minute.

RESULTS

Patient characteristics, responses, and durations of response are presented in table 1.

Measurable response occurred in ten patients, with three patients achieving a complete response (one of whom had disseminated disease in liver, bone, and lung). Evaluable response was seen in two of five patients. The overall response rate was 46%. Median duration of response for all responders is 6.5+ months (range, 2.5–19+). One patient in complete response has been off

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TABLE 1.—Patient characteristics

	No. of patients	Response*	Duration of response (mos)
Total			
Entered	26		
Evaluable	26	3 CR	12, 12+, 19+
		7 PR	2.5, 4, 4.5, 6.5+, 6.5+, 8, 11
		2 EVAL	3, 4
Median age in yrs (range): 67 (35-71)			
Sex (male/female)	15/11		
Performance status			
≥ 60 to < 80	10	2 PR	2.5, 4.5
		1 EVAL	4
≥ 80	16	3 CR	12, 12+, 19+
		5 PR	4, 6.5+, 6.5+, 8, 11
		1 EVAL	3
Histology			
Adenocarcinoma	15	2 CR	
		3 PR	
		1 EVAL	
Epidermoid carcinoma	6	2 PR	
		2 EVAL	
Large cell carcinoma	5	1 CR	
		2 PR	
Weight loss (≥ 10% of body weight)			
Yes	16	2 CR	12, 19+
		5 PR	2.5, 4, 4.5, 6.5+, 11
		1 EVAL	3
No	10	1 CR	12+
		2 PR	6.5+, 8
		1 EVAL	4
Extent of disease			
Localized	8	1 CR	12+
		3 PR	6.5+, 6.5+, 8+
		1 EVAL	3
Disseminated	18	2 CR	12, 19+
		4 PR	2.5, 4, 4.5, 11
		1 EVAL	4
Prior therapy			
Radiation therapy	6		
Chemotherapy	3		
Both	2		

* CR = complete response; PR = partial response; EVAL = evaluable response.

all chemotherapy for 7 months. Responses occurred in lung, liver, lymph node, and bone metastases. Median duration of therapy was three cycles of cisplatin (range, one to ten) and four cycles of mitomycin (range, one to 12).

Toxic effects included universal occurrence of nausea and vomiting. Because of gastrointestinal toxicity, four patients stopped cisplatin after an average of three cycles, including the one patient with a complete response after ten cycles. Hematologic toxicity included a median total wbc count nadir of 2400/μl (range, 1700-5600), with a neutrophil count nadir of 1400/μl (range, 300-3200). Median hemoglobin nadir was 9.8 g/dl (range, 8.0-14) and six patients required periodic transfusions because of progressive anemia. Thrombocytopenia of < 100,000 cells/μl occurred in only one patient. A serum creatinine rise above 1.5 mg/dl was noted in five patients. No patient stopped cisplatin or mitomycin because of nephrotoxicity. Twelve patients had grade 1 neurotoxicity (paresthesias) but no one required reduction of vinblastine dose due to neurologic side effects.

DISCUSSION

Recent trials in non-small cell carcinoma of the lung incorporating vindesine and cisplatin have shown a 40% re-

sponse rate, but the complete response rate remains small (1). A recent trial of vinblastine and cisplatin in 27 patients gave a 52% response rate, with a median survival of 22 weeks (6).

The current trial of MVP showed that the drug combination is effective (46% response rate; 95% confidence limits, 28.8%–64.5%) and well-tolerated. Of note is the rare occurrence of thrombocytopenia at this dose schedule despite continued mitomycin usage.

Because of the potential of greater toxicity with the three-drug combination, a prospective randomized trial of MVP versus vinblastine and cisplatin is appropriate.

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Bleomycin, Carmustine, Vincristine, and Dacarbazine in Patients With Metastatic Malignant Melanoma[1,2]

Seymour M. Cohen,* Takao Ohnuma, Tony Cheung, and James F. Holland[3]

Metastatic malignant melanoma has poor prognosis. In 1977, we reported a 42% response rate with a combination of carmustine, vincristine, and dacarbazine (BVD) (1). The duration of response in this study, however, was no greater than that achieved using the single agent dacarbazine. Because of the short duration of response and resistance of visceral metastases to this regimen, we designed a study to answer the question of whether a decrease in the interval of treatment from every 6 weeks to every 3 weeks and the addition of bleomycin to this modified regimen would improve the therapeutic effects.

PATIENTS AND METHODS

The characteristics of the 14 treated adult patients with metastatic malignant melanoma are shown in table 1. Patients were treated every 21 days with bleomycin (30 units sc), carmustine (75 mg/m^2 iv), dacarbazine (375 mg/m^2 iv), and vincristine (1 mg/m^2 iv [maximum unit dose, 2 mg]). The median number of treatment cycles was two (range, one to three cycles).

RESULTS

Of 14 evaluable patients, only one had a response: a patient with skin and nodal involvement showed a 75% regression of his metastatic axillary, supraclavicular, and cervical nodes lasting 6 weeks. There were no drug-related deaths, sepsis, bleeding, or pulmonary toxicity. All of the patients developed tolerable nausea and vomiting and the expected hematologic toxicity (table 1).

DISCUSSION

Bleomycin is an inactive drug in malignant melanoma when used as a single agent. De Wasch et al (2) originally reported a 48% response rate when lomustine and vincristine were combined with bleomycin. Considering the fact that lomustine and vincristine each used as a single agent gave remission rates of 13% and 12%, respectively, and that the combination of these two agents does not give a > 25% response rate, these results appeared impressive. Seigler et al (3) reported a 45% response rate with a combination of bleomycin, vincristine, lomustine, and dacarbazine, although a recent report following the completion of this study does not confirm the initial high response rate (4). In our experience, the BVD regimen is active; however, the response duration is brief and there are few complete responses. In addition, hepatic metastases are resistant to this regimen and there is a high rate of central nervous system relapse. In this study, we attempted to answer two questions: whether (a) adding

TABLE 1.—Patient characteristics and toxic effects

	No. of patients
Total	14
Male/female	8/6
Marrow function	
Wbcs > 4500/mm^3	14
Platelets > 100,000/mm^3	14
Disease sites	
Visceral	8*
Skin and nodal	5†
Osseous	2
Brain	1
Previous therapy	
Chemotherapy (dacarbazine)	3
Immunotherapy	0
Hematologic toxic effects	
Leukopenia	9
Nadir, 3000–4100/mm^3	8
Nadir, 300/mm^3	1
Thrombocytopenia	
Nadir, 75,000–100,000/mm^3	9

* 5 lung; 3 liver.
† 2 with additional visceral involvement.

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bleomycin and (*b*) increasing the frequency of drug administration would improve the response rate and the duration of responses. Since most of our patients in the previous BVD study had leukopenia and thrombocytopenia when the drugs were administered every 6 weeks, we decreased the carmustine dose when it was administered every 3 weeks.

One partial response in 14 patients leads us to conclude that the combination of bleomycin and BVD administered in this manner is inactive in malignant melanoma, with < 30% effectiveness at a probability of < 0.055. The reason for the inactivity of this regimen is unknown. It may possibly be attributed to the decreased carmustine dosage employed despite the increased frequency of administration. In the initial BVD study, we administered 65 mg/m² of carmustine daily for 3 days every 6 weeks, for a total of 195 mg/m². In contrast, in the current study, 75 mg/m² of carmustine was given every 3 weeks, for a total of 150 mg/m² in 6 weeks. Adequate drug dosage may be a crucial factor in the chemotherapy for malignant melanoma.

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Clinical Evaluation of 4-Demethoxydaunorubicin in Patients With Advanced Malignant Lymphoma[1,2]

Craig J. Coonley, Raymond P. Warrell, Jr,* David J. Straus, and Charles W. Young[3,4]

Anthracyclines are important drugs for the treatment of malignant lymphoma. 4-Demethoxydaunorubicin (DMDR) is a new anthracycline which is active by both oral and parenteral routes of administration (1). The drug produces substantially less cardiotoxicity in experimental animals relative to either doxorubicin or daunorubicin (2). After a phase I study at this center (3), we initiated a phase II trial of DMDR in patients with advanced malignant lymphoma.

Twenty-two patients who were refractory to conventional therapy were treated. Patient characteristics and major sites of extranodal disease are shown in table 1. Other extranodal sites included: bone (three patients); skin (two); and stomach, pancreas, bowel, breast, kidney, heart, and testis (one each). All patients had bidimensionally measurable disease, performance status ≥ 50 (Karnofsky), serum creatinine and bilirubin ≤ 1.5 mg/dl, and total wbc and platelet counts ≥ 3500 and ≥ 120,000/mm^3, respectively. Each patient had been treated with combination chemotherapy which included doxorubicin (table 1). Two patients had also received aclarubicin (100 and 120 mg/m^2, respectively). All patients but one had also received radiotherapy. Conventional response criteria were used (4).

DMDR was administered every 3 weeks at a dose of 12.5 mg/m^2 iv, with dose escalation by 2.5 mg/m^2 in the absence of toxicity. If hematologic toxicity occurred, subsequent doses were attenuated by 2.5 mg/m^2. The median dose of DMDR administered was 12.5 mg/m^2 (range, 6.25–17.5). The median number of doses administered was one (range, one to five doses).

No major antitumor effects were observed. Three pa-

TABLE 1.—Clinical characteristics of 22 patients treated with DMDR*

Median age in yrs (range)	43 (21–68)
Median Karnofsky performance status (range)	60 (50–90)
Median No. of chemotherapy agents (range)	8 (4–13)
No. of prior chemotherapeutic regimens (range)	3 (1–8)
Median cumulative dose of doxorubicin in mg/m^2 (range)	167 (32–500)
Major sites of extranodal involvement	
Marrow	4
Lung/pleura	6/4
Liver	4
Histologic diagnoses	
Hodgkin's disease	6
Diffuse poorly differentiated lymphocytic lymphoma	3
Diffuse large cell ("histiocytic") lymphoma	10
Nodular poorly differentiated lymphocytic lymphoma	1
Diffuse mixed lymphoma	1
Nodular mixed lymphoma	1

* Unless otherwise specified, values = No. of patients.

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tients with non-Hodgkin's lymphoma achieved minor responses (ie, < 50% decrease in the size of measurable disease) for 2, 3, and 3 months, respectively. Doses of DMDR ≥ 12.5 mg/m^2 were uniformly associated with mild nausea. The dose-limiting toxic effect was myelosuppression. The median wbc count nadir was 1800/mm^3 (range, 100–4900) and the median platelet count nadir was 86,000/mm^3 (range, 11,000–289,000). Six patients (27%) required hospitalization for fever related to neutropenia and two patients required platelet transfusion for a platelet count < 20,000/mm^3. Five patients developed mucositis of mild degree. No adverse cardiac effects were noted. The highest cumulative dose received by any single patient was 62.5 mg/m^2.

Although our patients had received extensive prior therapy, six subsequently had major responses to other therapy. In a similar patient population treated with a related anthracycline (aclarubicin), we noted major responses only in patients who had not previously received doxorubicin (5). Thus, it appears likely that both of these new agents share clinical cross-resistance with standard anthracyclines. We cannot recommend further investigation with DMDR in similar patient populations using this dose and schedule. Future studies of this agent should be conducted in patients who have not received treatment with other anthracyclines.

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Etoposide in Small Cell Lung Cancer Resistant to Prior Chemotherapy[1,2]

Robert F. Asbury,* Jonathan Rubins, and John M. Bennett[3]

Etoposide (VP-16-213) is generally recognized as one of the most active single agents against small cell carcinoma of the lung (1). Pooled data from phase I and II studies with previously treated patients produced a 33% response rate (2,3). The University of Rochester Cancer Center has treated 29 patients with VP-16-213 in the hope of confirming these early reports of efficacy in this setting.

Patients received 140 mg/m^2 iv on Days 1, 3, and 5, with the cycle repeated in 4 weeks as recommended by the Division of Cancer Treatment, National Cancer Institute, for group C guideline drugs. Dose escalations of 20% were used if no leukopenia or thrombocytopenia was seen with nadir counts. Doses were decreased by 33% if nadir wbc counts were < 2000/mm^3 or if nadir platelet counts were < 75,000/mm^3.

Twenty-nine patients were entered in this study. Four patients were not evaluated. Patient characteristics are shown in table 1. All patients had progressive disease during treatment with combination therapy. Twenty-five patients had received treatment with cyclophosphamide, lomustine, and methotrexate or with this combination alternating cycles with doxorubicin, vincristine, and procarbazine. Four patients had received therapy with cyclophosphamide, doxorubicin, and vincristine. Despite this heavy prior therapy, the median performance status for the group was 1.5 (Eastern Cooperative Oncology Group [ECOG] criteria; range, 1-3).

There were no complete or partial remissions in any of these patients. Three patients had disease stabilization for 4, 5, and 8 months. Four patients are alive at 4, 5, 5, and 8 months.

Toxicity was mild in most patients. The average dose received for the group was 102% of the starting dose. Nausea and vomiting were mild and caused dose reduction in only two patients. There were two drug-related deaths due to pneumonia and leukopenia in patients with postobstructive infiltrates prior to VP-16-213.

We found VP-16-213 to be ineffective therapy for patients with small cell carcinoma of the lung progressing on modern multi-agent chemotherapy. It is therefore more appropriate for use in first-line combination therapy. This study confirms results of other previous trials and indicates that VP-16-213 has little value in this setting (4).

TABLE 1.—Patient characteristics and response[*]

Sex	
Male	14
Female	15
Average age in yrs	57
Performance status (ECOG)	1.5
Prior combination chemotherapy	29
No. of chemotherapy courses/patient	1.7
Evaluation for response	25
Wrong diagnosis	1
Died within 1 wk	2
Received VP-16-213 in combination	1
Stage	
Locoregional	6
Disseminated	19
Brain	5
Liver	13
Bone	7
Other	4
Bone marrow toxicity	
Median wbc count nadir (range), cells/mm^3	2500 (500-5100)
Median platelet count nadir (range), cells/mm^3	90,000 (23,000-350,000)
Recovery time in days	21
Response	
Complete	0
Partial	0
Stable disease	3 (4, 5, and 8 mos)
Alive	4 (4, 5, 5, and 8 mos)
Average survival in mos	3.4+

[*] Unless specified otherwise, values = No. of patients.

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Phase II Study of Oral Medroxyprogesterone Acetate in Advanced Germ Cell Tumors[1,2]

F. Cavalli,[3,*] O. Klepp,[4] H. Hansen,[5] and M. Rozencweig[6]

Medroxyprogesterone acetate (MPA) induces testicular atrophy and inhibits spermatogenesis in animals (1). The presence of androgen receptors has been recently demonstrated in normal and malignant human testicular tissues (2,3). Progesterone may inhibit the binding of androgens to cytosol receptors (3). These observations suggest that hormonal manipulations might be of potential therapeutic value in testicular cancer, which is further substantiated by the objective improvement that has been observed in three of 16 patients with metastatic teratoma treated with oral MPA (4). These considerations led us to initiate a phase II trial with MPA in patients with advanced germ cell tumors.

PATIENTS AND METHODS

Twenty patients with advanced, measurable germ cell tumors were entered in the study (table 1): 14 had nonseminomatous testicular cancer, three had testicular seminoma, one had extragonadal seminoma, and two had extragonadal nonseminoma. Patients with positive markers as sole evidence of disease were not eligible. MPA (500 mg/m^2/day orally) was administered until disease progression occurred. Drug effect was assessed according to World Health Organization (WHO) criteria.

RESULTS

None of the patients met the response criteria. One patient with extragonadal seminoma had stable disease for 2 months before showing disease progression. Toxic effects from MPA were minimal and never required alterations in the dose schedule or drug withdrawal.

DISCUSSION

The study failed to demonstrate any antitumor efficacy with oral MPA in the treatment of advanced germ cell tumors. Therefore, we feel that further studies with this compound in germ cell tumors are not warranted, even if the patient population entered in our trial represented a poor-risk group and the therapeutic response to MPA might be dose-dependent (5).

TABLE 1.—Characteristics of 14 patients with nonseminomatous testicular cancer

Characteristic	No. of patients
Median age in yrs (range): 27 (18–50)	
Median performance status (range): 1 (0–3)	
Previous chemotherapy	
3–4 drugs	3
5–6 drugs	10
> 6 drugs	1
Previous radiotherapy	
None	6
Limited	3
Extensive	5
Serum markers	
Unknown	2
Negative	2
Positive*	10
Metastatic sites	
Lung	13
Abdominal mass	5
Extra-abdominal nodes	4
Soft tissue	2
Chorioidea	1
Histologic subtype	
Embryonal carcinoma	4
Embryonal carcinoma + seminoma	2
Embryonal carcinoma + sinus endodermal	2
Embryonal carcinoma + choriocarcinoma	2
Teratocarcinoma	3
Teratocarcinoma + seminoma	1
Median duration of treatment in days (range): 42 (18–119)	
Median survival from start of treatment (days): 60	

* At least 1 (β-HCG, α-fetoprotein).

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[2]Conducted by the Early Clinical Trials Group of the European Organization for Research on Treatment of Cancer (EORTC).

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Evaluation of Mitolactol in Women With Advanced Breast Cancer and Prior Chemotherapy Exposure[1,2]

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Mitolactol (DBD) is a halogenated hexitol which appears to act as an alkylating agent (1). Andrews et al (2) used a daily oral schedule of DBD to treat 33 patients with breast cancer; one had a complete response and 12 had partial or mixed responses. Details of prior treatment were not given. Tormey et al (3) developed an intermittent oral schedule of DBD and recommended a dose of 180 mg/m^2 daily for 10 days every 28 days in patients with prior treatment. Three partial regressions were seen in 29 patients with breast cancer entered in this phase I–II study. The following clinical trial was performed to evaluate the activity of DBD in women with advanced breast cancer who had received multiple prior chemotherapy regimens.

PATIENTS AND METHODS

Twenty-four women with histologic confirmation of breast cancer and progressive metastatic disease were entered in this study (NCCTG 79-32-51) (table 1); two were excluded because of ineligibility. Eligibility requirements included prior treatment with a first-line combination chemotherapy regimen and treatment with single-agent doxorubicin as the regimen immediately preceding DBD therapy. Patients were required to have a wbc count $\geq$ 4100/mm^3, a platelet count $\geq$ 130,000/mm^3, creatinine $<$ 2.0 mg/dl, and total bilirubin $\leq$ 2.0 mg/dl. DBD was administered orally at a daily dose of 180 mg/m^2 for 10 consecutive days, with a planned cycle length of 28 days. Response criteria employed were as previously reported for measurable (4) and evaluable (5) disease.

RESULTS

No objective regressions were observed in the 22 evaluable patients. Fifteen (68%) patients had disease progression by the 1-month assessment. An additional patient who was stable at 1 month had persistent myelosuppression and subsequently had disease progression before return of blood cell counts to a level acceptable for re-treatment. The median survival for all patients was 97 days; 21 (95%) patients have died. The main toxicity encountered was hematologic. The median time to platelet count nadir was 28 days, which was the planned cycle length, but this is an underestimate of the median since some patients had no counts obtained beyond this time. Of the six patients receiving more than one cycle of therapy, four required delays of 7, 12, 14, and 34 days in initiation of Cycle 2 because of prolonged count suppressions. Nonhematologic toxicity was mild, with 36% of the patients reporting nausea and 18% reporting emesis.

DISCUSSION

The finding of no objective regressions in 22 patients is inconsistent at the 0.05 level with a true regression probability of $\geq$ 0.13, and it is concluded that the intermittent regimen of DBD used does not have appreciable activity in patients with breast cancer with prior multiple regimen exposure. From a toxicity standpoint it does appear that a planned cycle length of 28 days with the intermittent schedule is inappropriately short because of the observed median time to platelet count nadir.

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[2]This study was conducted as a collaborative trial of the North Central Cancer Treatment Group and the Mayo Clinic and was supported in part by Public Health Service grant CA-25224 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services. The following institutions and members participated in the study: Mayo Clinic, Rochester, MN (*J. N. Ingle, S. J. Green,* and *L. K. Kvols*); Iowa Oncology Research Association, Des Moines (*S. F. Brunk*); Duluth Clinic, MN (*J. E. Krook*); and Fargo Clinic, ND (*L. K. Everson* and *P. S. Etzell*). Also participating were: Grand Forks Clinic, ND (*J. Laurie*); Quain and Ramstad Clinic, Bismarck, ND (*D. Pfeifle*); and St. Cloud Internists, Ltd, MN (*H. Windschitl*).

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TABLE 1.—Clinical data

Characteristic	No. of patients
Evaluable	22
Age (yrs)	
Median: 57	
Range: 38–79	
Dominant disease status	
Soft tissue	3
Osseous	6
Visceral	13
Sites of disease	
Soft tissue	16
Osseous	15
Lung	8
Pleura	3
Liver	5
No. of sites	
1	6
2	8
3	7
5	1
Eastern Cooperative Oncology Group performance status	
0	5
1	8
2	7
3	2
Prior hormonal therapy	13
Prior chemotherapy*	
CFP	19
CMF	4
Doxorubicin	22
No. of prior chemotherapy regimens	
2	18
3	3
4	1
Response to prior chemotherapy	10
Indicator lesion	
Measurable	18
Evaluable	4
No. of DBD treatment cycles	
1	16
2	3
3	2
5	1
Hematologic toxicity†	
Wbc count nadir (cells/mm^3)	
<4100	12
<3000	7
<2000	3
<1000	3
Median (range): 3900 (300–8700)	
Median time to nadir (range): 20 days (4–45)	
Platelet count nadir (cells/mm^3)	
<130,000	11
<100,000	11
<50,000	6
<20,000	4
Median (range): 94,000 (4000–280,000)	
Median time to nadir (range): 28 days (19–38)	

*C = cyclophosphamide, F = 5-FU, M = methotrexate, and P = prednisone.

†Cycle 1: 19 evaluable patients.

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High-Dose Etoposide as Single-Agent Chemotherapy for Small Cell Carcinoma of the Lung[1]

Etoposide is one of the more active agents in the treatment of small cell carcinoma of the lung (SCCL). The standard dosage of this drug is 250–500 mg/m² every 2–3 weeks, yielding response rates of 40%–50% for SCCL (1,2). Dose augmentation studies have administered etoposide at up to 1500 mg/m², with myelosuppression and mucositis notable (3,4). At Vanderbilt University, using autologous bone marrow transplantation to assure reconstitution of hematopoiesis, etoposide has been administered at doses up to 2400 mg/m², with severe mucositis limiting further increments (5).

In an attempt to explore a possible dose-response relationship, 1200 mg/m² (400 mg/m²/day for 3 consecutive days) of etoposide was given as initial therapy to 13 patients with extensive-stage SCCL in a prospective trial between January and August 1981 (table 1). This dose was chosen to be one-half of the maximally tolerated dose, representing a significant increase over standard dose, but not expected to be associated with prohibitive toxicity. Two cycles of etoposide at 1200 mg/m² at 3–4-week intervals were used as initial therapy. Etoposide was then followed by six cycles of conventional combination chemotherapy with cyclophosphamide, doxorubicin, and vincristine (CAV). Complete responders received late consolidation therapy with 1200 mg/m² of etoposide after completion of CAV therapy. Partial responders were to receive radiotherapy to sites of persistent disease. Antitumor evaluation was performed after the initial two cycles of etoposide.

Three patients died during the initial course of therapy: one of hepatic failure secondary to liver involvement with tumor, one of respiratory failure secondary to obstructive lung disease and tumor, and one of staphylococcal sepsis causing toxic epidermal necrolysis. All three patients were bedridden and died within 1 week of initiation of chemotherapy, and all were neutropenic and febrile at the time of death. Antitumor evaluation was difficult to ascertain in these patients.

Of the remaining ten patients, four had a complete response, four had a partial response, and two failed to respond to etoposide therapy. Despite additional conventional therapy, none of the partial responders had further antitumor improvement. Late consolidation with etoposide was given to two of the four complete responders. Forty-two percent of the courses of etoposide were associated with fever and neutropenia, with a median nadir wbc count of 600/μl (range, 80–2100) and a median nadir platelet count of 23,000/μl (range, 6500–136,000). All patients have had tumor recurrence or progression. For all 13 patients, median survival was 7.5 months (range, 0–14+). Three of the five nonambulatory patients died during therapy, and all ambulatory patients tolerated therapy adequately.

TABLE 1.—Patient characteristics (N = 13)

Characteristic	
Median age in yrs at diagnosis (range)	56 (36–74)
% of males/females	77/23
Performance status	
% ambulatory	62
No. of patients	
Sites of metastases	
Liver	8
Bone, bone marrow	8
Pleural effusion only	1
Lymph node only	1
Previous treatment (total)	2
Chemotherapy	2
Radiation therapy	2

In this trial, eight of 13 patients responded to etoposide at a dose of 1200 mg/m², with a complete response rate of 31% and a partial response rate of 31%. Although the response rate in this small series is not significantly different than the rate reported with standard doses of etoposide, observations of complete response in extensive-stage patients to single-agent therapy appear provocative. High-dose etoposide, although more myelosuppressive, is now being evaluated in a large prospectively randomized study to corroborate a dose-response relationship. If increased antitumor activity of high-dose etoposide is confirmed, it should be added to effective agents as front-line therapy for SCCL.

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Neurotoxicity of Interferon

Interferon (IFN) is known to enhance the excitability of neurons in vitro (1). Confusion, fatigue, weakness, and lethargy, as well as mild paresthesia (2), have been reported in patients receiving IFN, but the mechanisms of these untoward effects have not been clarified.

The blood-brain (3) and brain-cerebrospinal (CSF) (4) barriers for IFN are strong. High doses of iv or im IFN are needed to achieve measurable levels in CSF. In monkeys, only small amounts of IFN penetrated to the deep layers of the brain after intrathecal administration, and no anatomopathologic changes indicative of local toxic effects could be demonstrated in arachnoids, spinal cord, or brain. A. Lister (personal communication, 1982) noted EEG changes, including generalized slowing in patients receiving high iv doses of lymphoblastoid IFN, but no neurophysiologic documentation of IFN neurotoxicity has been published so far.

We observed severe but reversible neurotoxicity that was also documented by characteristic alterations in EEG and electroneuromyography (ENMG) after high-dose iv infusion of human leukocyte IFN in patients with lung cancer. These patients (five men and three women, 53–75 yrs of age [mean, 64]) had small cell bronchogenic carcinoma confined to one hemithorax. All patients had good initial performance status (Karnofsky scale 80%–100%), and all were previously untreated. The patients received treatment in the Department of Thoracic Medicine and were assessed daily by senior staff members and a radiation oncologist. In addition, two of the eight patients were prospectively followed by a neurologist and a neurophysiologist. The effect of IFN on neurologic and psychic functions was evaluated from clinical and laboratory tests, including EEG and ENMG registrations, neurologic examination, and from observations on speed and accuracy of speech, state of consciousness, serial handwriting tests, mood, and overt behavior. The tests and evaluations were performed before treatment on Days 1, 3, and 5, weekly for 4 weeks, and then monthly. In addition, temperature, blood pressure, and all signs or symptoms were recorded every 2–4 hours during the 5-day period of iv infusion.

Six patients received partially purified human leukocyte IFN (P-IF B+A (5), specific activity $1–3 \times 10^6$ IU/mg protein) and two received highly purified IFN (NK2IFN, specific activity $1–2 \times 10^8$ IU/mg protein), obtained by passage through a monoclonal antibody affinity chromatography column (NK2 Sepharose, Celltech, England). The planned dose to be administered on Days 1 and 2 was 100×10^6 IU/24 hours, followed by 200×10^6 IU/24 hours on Days 3–5, or a total of 800×10^6 IU/5 days as continuous iv infusion (clockwork or AGA IVAC 564 pump). The iv treatment was to be followed by a low-dose maintenance treatment of 6×10^6 IU given im three times per week, commencing on Day 8, and continuing until systemic non-CNS relapse of the disease, when conventional combination chemotherapy was started.

Four patients received the total planned dose of 800×10^6 IU/5 days; for three patients, the dose was reduced to 425, 500, and 700×10^6 IU, respectively, due to changes in hematologic parameters. For one patient, the dose had to be reduced to 680×10^6 IU because of severe hypovolemic, not IFN-related, shock. Care was taken to supply the patients with sufficient hydration and nutrition throughout the infusion period. In all patients, maintenance treatment was started within 2 weeks from the end of the high-dose phase. The mean duration of treatment with IFN alone was 18 weeks (range, 5–42), and with IFN plus concomitant radiotherapy, 25+ weeks (range, 5–44+).

Fever with severe shaking during the peaks, malaise, and muscle pain occurred in all patients a few hours after the start of the infusion and lasted throughout the iv infusion. On Day 2 of the infusion, the patients became excited, almost euphoric. Thereafter, a progressive mental and motor slowing set in. Great fatigue, somnolence, and lack of initiative were characteristic. Speech became unclear and inaccurate, and only slow answers could be obtained. The patients were unable to complete self-assessment forms, and their handwriting changed. Perseveration was characteristic, and loss of smell and taste was typical. Serial EEG recordings showed progressive general slowing of background activity from alpha to

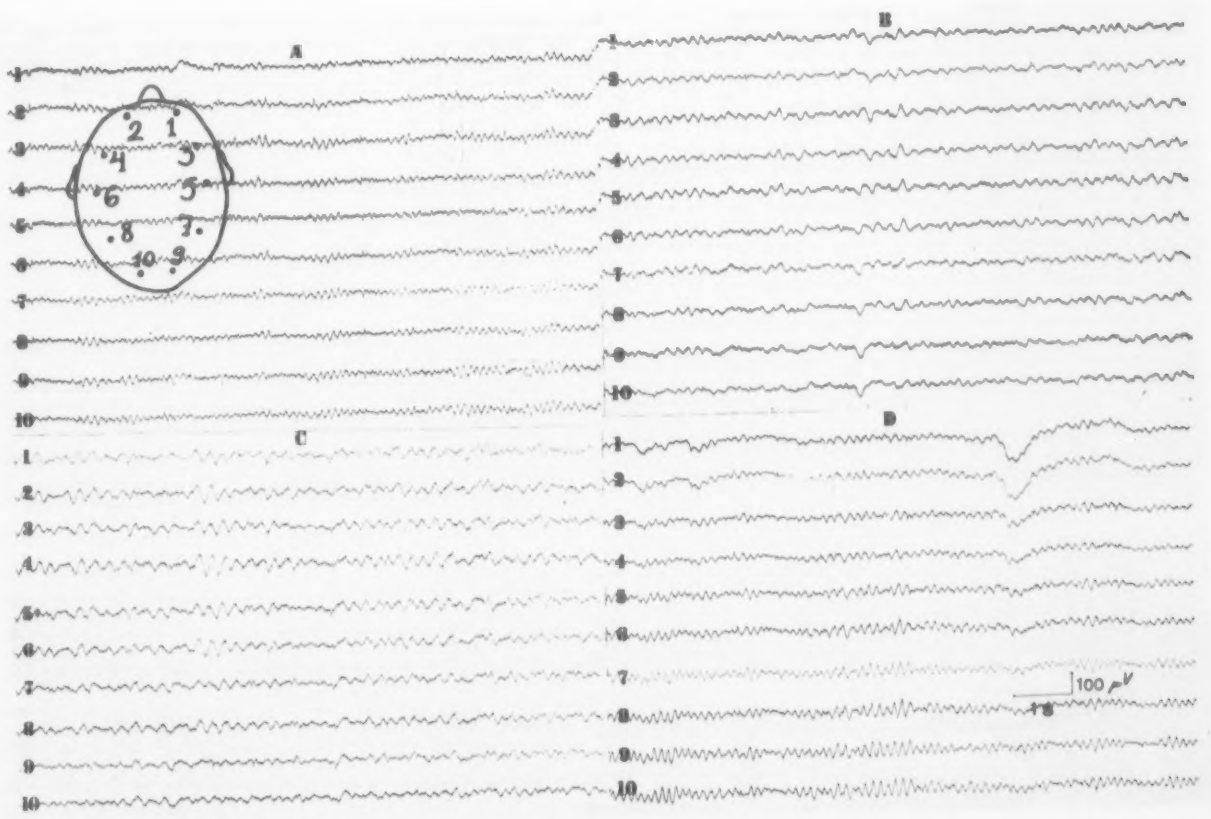

FIGURE 1.—Serial EEG of a 60-yr-old woman with small cell lung cancer: A = before IFN treatment—8–9 Hz alpha rhythm; B = Day 3 of high-dose iv treatment—the dominant activity is 4–6 Hz with low-voltage delta waves in the frontal areas, and very little alpha rhythm remains in the occipital region; C = Day 12, at end of 1st wk of im treatment—marked general slowing without normal alpha rhythm; D = Day 26, at end of 3rd wk of im treatment—the slowing has completely disappeared, and dominant activity is normal alpha rhythm as in the pretreatment recording.

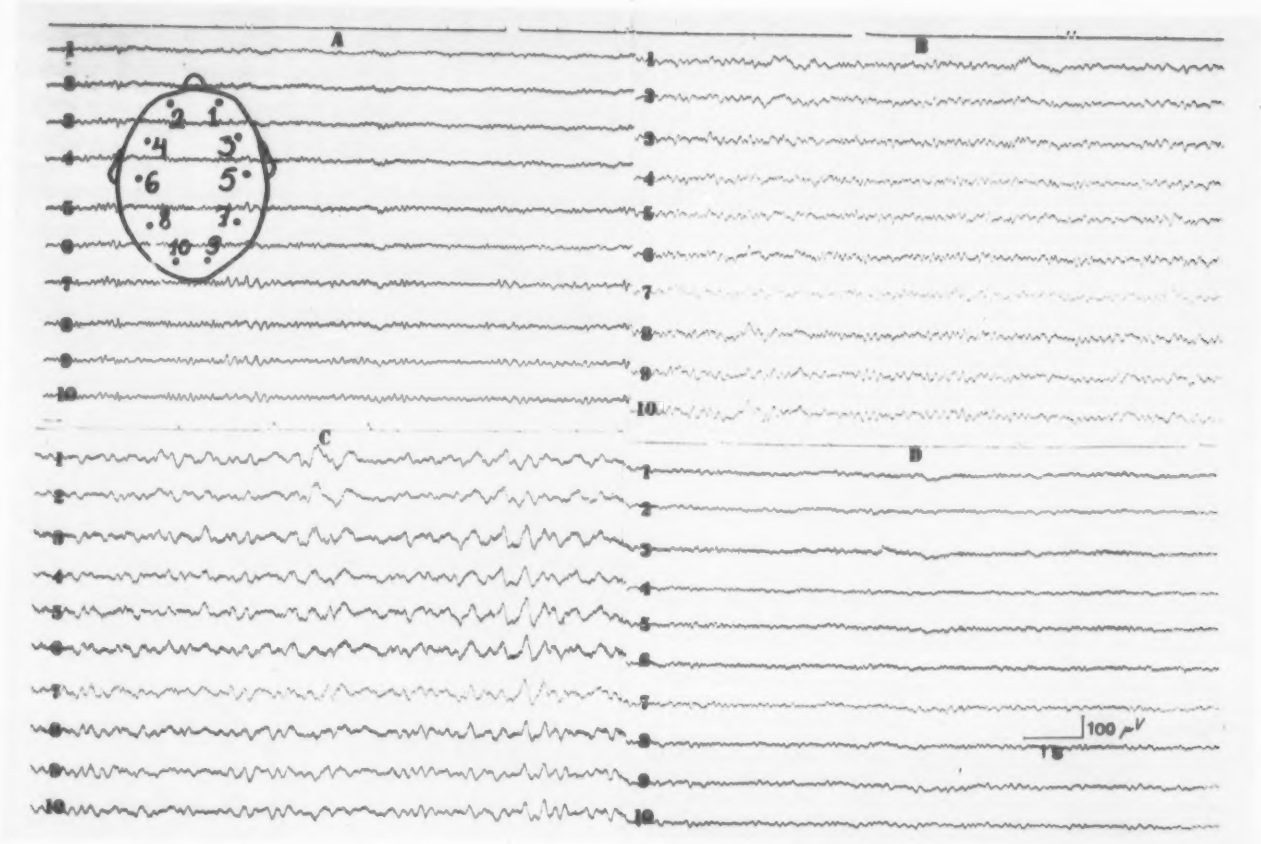

FIGURE 2.—Serial EEG of a 75-yr-old man with small cell lung cancer: A = before IFN treatment—slight abnormalities with short trains of 8 Hz waves on the dominant 10–11 Hz alpha rhythm; B = Day 2 of high-dose iv treatment—background activity is 9 Hz, and low polymorphic theta-delta waves are seen mainly in the middle and occipital regions; C = Day 12, at end of 1st wk of im treatment—marked general slowing of theta-delta range without normal alpha rhythm; D = Day 26, at end of 3rd wk of im treatment—the slowing has completely disappeared, and dominant activity is normal alpha rhythm as in the pretreatment recording.

theta range, with diffuse delta waves predominantly in the frontal lobe (figs 1 and 2). Derangement of peripheral nervous function was expressed by loss of tendon reflexes, mild to marked motor loss, and slowing of motor and sensory conduction velocities on ENMG. Sensibility was unaffected.

Tests for antibodies against IFN in serum have remained negative. Only with the high iv dose ($100–200 \times 10^6$ IU/24 hours) did CSF contain measurable amounts of IFN (35–110 IU/ml). Lumbar puncture samples assayed during the course of the low-dose phase were all negative for IFN. Differential cell counts showed that an increase in the proportion of enlarged lymphocytes in CSF coincided with EEG abnormalities. No patients had an anaphylactoid reaction to IFN. Steroids were not used as symptomatic treatment.

The neurophysiologic, clinical, and laboratory abnormalities gradually subsided within 2–4 weeks after the end of the high-dose phase while low-dose IFN treatment continued. Side effects with partially and highly purified IFN were indistinguishable. Thus, the observed neurologic effects were probably caused by interferon and not by impurities in the IFN preparations.

These observations suggest that adverse neurologic effects are a dose-limiting factor in high-dose IFN treatment, and raise the possibility that endogenous IFN plays a role in the pathogenesis of virus-induced encephalopathy and neuropathy.

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November 18, 1982

The National Cancer Institute Clinical Investigator Award

Continuous application receipt dates: June 1, October 1, February 1

The National Cancer Institute announces the availability of Clinical Investigator Awards. The initiation of this award is intended to encourage recently-trained highly-qualified physicians to undertake careers in cancer research. The award is prompted by the chronic shortage of investigators particularly in surgical oncology, radiation oncology, preventive oncology, physiatry, nutrition, and epidemiology. It is expected to facilitate the awardees' transition to an independent basic or applied researcher. The award provides three years of support for successful candidates. Support is based upon a full-time, twelve-month staff appointment. Recipients of the award will receive salary support not to exceed $30,000 annually from NCI funds for the three-year period. Up to a total of $10,000 annually will be provided for supplies, equipment, travel, etc, which are deemed necessary for the research program.

To be eligible for an award, applicants must identify an established investigator to serve as a mentor. This sponsor must make a commitment to supervise and guide the candidate in his/her research. A well-detailed description of the research plan must be provided by the candidate in the application and evidence of institutional support must also be clearly demonstrated. Recipients must also agree to spend at least 75% of their time pursuing the research project proposed in the application for this training grant. Up to 25% of the grantee's time may be spent involved in teaching and/or other clinical activities relevant to their career development. Grants will be awarded to the recipient's parent institution and institutions will be allowed to apply for awards on behalf of individuals.

For more information, contact: Barney C. Lepovetsky, Chief, Cancer Training Branch, Division of Resources, Centers, and Community Activities, Blair Bldg, Rm 717, 8300 Colesville Rd, Silver Spring, MD 20910 (telephone 301-427-8898).

Current Concepts in Cancer Therapy, II

St. Louis, Missouri, November 3–5, 1983

This meeting will be presented by the Department of Surgery and the Office of Continuing Medical Education, Washington University School of Medicine in St. Louis. The program chairman is Marc K. Wallack, MD. The meeting will be accredited for 17 hours AMA Category 1, AAFP Prescribed, and AOA.

For further information, contact: Loretta Giacoletto, Office of CME, Washington University School of Medicine, Box 8063, 660 S Euclid, St. Louis, MO 63110 (telephone 314-454-3873).

International Symposium on Gynecologic Oncology, Surgery, and Urology

Miami, Florida, November 14–16, 1983

This symposium, designed to bring together gynecologists who are in surgical gynecologic practice with primary interest and experience in major gynecologic cancers, is sponsored by the University of Miami School of Medicine, Department of Obstetrics and Gynecology. This is not a review course, but rather an attempt to bring together specialists from different countries with divergent backgrounds to stimulate lively discussions on each topic. The presentations by the University of Miami faculty are designed to provide recent data and current clinical protocols to open discussion on controversies in selected aspects of their specialty. The program will be presented in English, German, and Spanish.

This course meets the criteria for 18 credit hours in Category I of the Physician's Recognition Award of the American Medical Association, and for 18 cognates, Formal Learning, by the American College of Obstetrics and Gynecologists. Tuition is $225.

For more information, contact: University of Miami School of Medicine, Department of Obstetrics and Gynecology R-116, PO Box 016960, Miami, FL 33101 (telephone: 305-547-6944 or OB-GYN Seminars 305-251-0591).

Position Vacancy: Associate Director, Radiation Research Program

The position of *Associate Director of the Radiation Research Program* is now vacant. This position is located within the Division of Cancer Treatment, National Cancer Institute (NCI), National Institutes of Health, Department of Health and Human Services.

This is a civil service position in the Senior Executive Service (SES), with a salary range from $56,945 to $63,800 per year. Candidates may be eligible for Physicians Comparability Pay up to $10,000 per year. (Alternatively, candidates may be eligible for appointment in the Commissioned Corps of the US Public Health Service). The individual selected, if not presently in SES, must serve a 1-year probationary period.

The Associate Director for the Radiation Research Program has overall responsibility for the planning, evaluation, and coordination of broad national and international radiation research programs designed to determine the effectiveness of radiation in cancer treatment, usefulness in diagnosis of cancer and other diseases, and biological effects of exposures to low doses of ionizing and non-ionizing radiation. The Associate Director also administers a $60 million contract and grant program.

Applications (personal qualifications statement SF-171) are to be sent to the address below. *Applications must be postmarked by November 15, 1983.* A current curriculum vitae and bibliography must accompany all applications. The National Institutes of Health is an Equal Opportunity Employer.

For further information, contact: Cynthia A. Kauff, PMS, National Cancer Institute, Bldg 31, Rm 3A23, Bethesda, MD 20205 (telephone: 301-496-6864).

Association for Brain Tumor Research Applications for 1984 Fellowships

The Association for Brain Tumor Research (AFBTR), Chicago, is now accepting applications for fellowships from postdoctoral candidates whose intent is to pursue a career in brain tumor research. Preference will be given to MD's who are citizens or permanent residents of the US seeking research training that will enable them to pursue careers as clinical investigators.

AFBTR fellowships offer an award of $18,000 per annum plus a $2000 annual travel allowance for a period of two years, with a third year renewable option. Each year's travel must include one presentation to the AFBTR Board of Directors in Chicago.

All applications will be reviewed by the Association's Medical Advisory Council and awards will be made based on their recommendations. Criteria for selection will include the quality of the individual applicant; the quality of the program to which he/she is applying; and the research he/she is undertaking. Applications must be submitted by the sponsoring program director no later than *January 6, 1984*. Awards will be announced by April 1, 1984, for fellowships to begin July 1, 1984.

Application forms may be obtained from and completed applications should be mailed to: The Association for Brain Tumor Research, Suite 200, 6232 N Pulaski Rd, Chicago, IL 60646.

For further information, contact: Cynthia A. Kauff, PMS, National Cancer Institute, Bldg 31, Rm 3A23, Bethesda, MD 20205 (telephone: 301-496-6964).

Second International Conference on Human Tumor Markers

Vienna, Austria, February 20–22, 1984

This conference is sponsored by the International Society for Preventive Oncology (ISPO), the Austrian Cancer Society-Austrian Cancer League, and the Association of Clinical Scientists, USA. Sessions, structured as workshops, include an update on biological and clinical applications of tumor markers for risk assessment, cancer detection, and patient surveillance. Podium and poster presentations, panel discussions, and scientific and technical exhibits are scheduled. Registration includes round table luncheon discussions.

Abstracts of presentations on relevant topics are invited by *September 15, 1983,* which will appear in *Cancer Detection and Prevention* vol 7(1), 1984. An award for original communication of outstanding investigation(s) will be presented by ISPO.

For further information, contact: Human Tumor Markers Conference, AMEXPOB # 790459, Dallas, TX 75379 (telephone: In US, 1-800-527-0297; Outside Europe, [usa-214]392-3663; In Europe, [austria 43-222] 52-0544).

Intra-Arterial and Intracavitary Chemotherapy 1984

San Diego, California, February 24–25, 1984

This continuing medical education conference will take place at the Holiday Inn at the Embarcardero (San Diego, CA). The conference is approved for 14 hours of AMA/CMA credit as well as AAFP and the fee is $250.

For more information, contact: Office of Continuing Medical Education, UCSD School of Medicine, M-017, La Jolla, CA 92093.

Cancer Treatment Symposia: Proceedings of the United States-Japan Meeting on Drug Development and Cancer Treatment Research

Limited copies of this first issue of *Cancer Treatment Symposia* (Volume 1, 1983) are available without charge from the Division of Cancer Treatment, National Cancer Institute.

The US-Japan Cooperative Cancer Research Program, sponsor of the US-Japan meeting on Drug Development and Cancer Treatment Research (Gettysburg, PA, Nov 1981), is an important program in a series of bilateral agreements developed by the National Cancer Institute with other countries to encourage and support intensive international collaboration in cancer research. The Proceedings of this meeting document the recent exchange of information between Japan and the United States on the current status of new anticancer drug development in the two countries.

For a copy of this issue of Cancer Treatment Symposia, contact: Anne McCarthy, Editor, Cancer Treatment Symposia, National Cancer Institute, R. A. Bloch International Cancer Information Center, Bldg 82, Rm 229, National Institutes of Health, Bethesda, MD 20205 (telephone: 301-496-6975).

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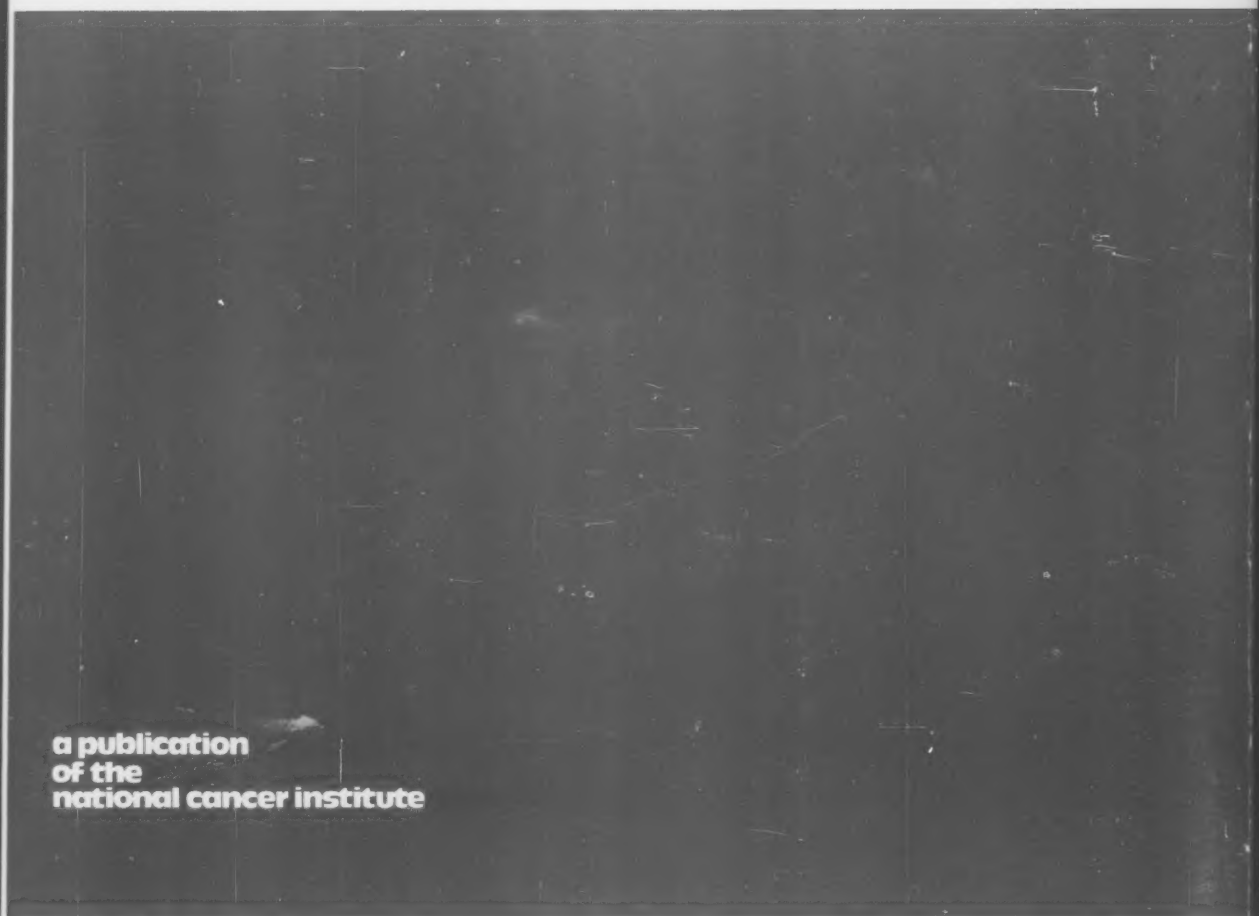

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